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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/10, C12Q 1/68, C12N 15/66

A1

(11) International Publication Number: **WO 98/38297**

(43) International Publication Date: 3 September 1998 (03.09.98)

(21) International Application Number: PCT/IL98/00095

(22) International Filing Date: 26 February 1998 (26.02.98)

(30) Priority Data:
120338 27 February 1997 (27.02.97) IL

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SINGLE STEP ASSEMBLY OF MULTIPLE DNA FRAGMENTS

(57) **Abstract**

A method is described for the efficient single step directional assembly of two or more DNA fragments into a construct whereby fragments with complementary ends of at least 15 nucleotides are amplified by PCR or obtained by restriction cleavage, single–stranded overhangs are generated utilizing exonucleolitic activity of exonuclease III or T4 DNA polymerase or a functional equivalent thereof; after expose of the overhangs, the fragments are subjected to conditions facilitating annealing of the complementary overhangs, thus creating a single functional construct, then, the gaps which may have been created by the prior action of the exonuclease treatment, in the adjoining regions of the said construct are filled–in by T4 DNA polymerase or T7 DNA polymerase or by a functional equivalent thereof, and the construct is transformed and/or transfected into host cells with or without a prior ligation step.

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SINGLE STEP ASSEMBLY OF MULTIPLE DNA FRAGMENTS

Field of the Invention

The present invention concerns an improved method for joining a number of DNA fragments into a multi-DNA fragment assembly in a desired predetermined directional manner in which method the actual joining of the DNA fragments is essentially a single step. More specifically, the present invention concerns an improved method for combining DNA fragments having regulatory function, such as, for example, promoters and the like, and DNA fragments encoding various proteins, such as, for example, enzymes, cytokines, hormones and the like, into a single construct for the purpose of cloning and expression of such a construct in a simple and rapid procedure, by way of which the various DNA fragments are connected in a predetermined direction and after joining of the fragments, there is no unwanted linker DNA between the joined fragments. Accordingly, the method of the present invention provides also for a seamless joining of DNA fragments.

Background of the Invention

Building DNA constructs is the core of genetic engineering. Building complex constructs requires time, money and highly skilled personnel. The construction is performed by fusing together specific DNA fragments in a desired way. The state of the art, as concerns cloning DNA fragments, has been published in a very large number of books, articles, patent applications, patents, and the like, and is usually readily available and known to all of skill in the art. For example, a comprehensive account of DNA cloning procedures is provided in the three volume text by Sambrook et al. (1989) entitled "Molecular Cloning - a Laboratory Manual", 2nd Edition, Cold Spring Harbor Laboratory Press. This extensive account of the prior art techniques for the combination of DNA fragments, cloning and expression thereof, is included herein by reference, in its entirety.

In accordance with the prior art, each of the DNA fragments is initially cleaved from larger DNA entities (cDNA, genomic DNA) with enzymes called restriction enzymes. The desired fragments are then covalently connected together by use of a ligase enzyme.

A given restriction enzyme is able to cleave DNA at a specific short sequence known as a restriction site. With more than two hundred different restriction enzymes which are commercially available, restriction sites situated at random up and downstream of a desired gene (or any other sequence of choice on a given DNA entity), can be identified and then cleaved by the respective enzymes. The DNA sequences which constitute restriction sites are mostly palindromic, between four to eight base pair long. Most enzymes cleave the DNA in the middle of the restriction site, leaving either "blunt" or "staggered" ends, depending on the specific enzyme. DNA with a "staggered" end has a short stretch (also known as "overhang") of single-stranded DNA between two to four bases long.

The enzyme ligase can connect, or ligate, two blunt ends of two DNA fragments (each fragment is a separate molecule) and form one longer fragment (longer molecule). Such a ligation is extremely inefficient. Ligase can also ligate two molecules with staggered ends if the overhangs of these two molecules are complementary to each other. In fact, matching overhangs greatly improve ligation between molecules: the single-stranded DNA on one molecule has an affinity to the complementary single-stranded DNA on the other molecule. The staggered ends, also known as "sticky ends", form non-covalent connections with one another via hydrogen bonds. Since the overhangs produced by restriction enzymes are short, these connections are weak and unstable, as the two molecules are held together by only a few hydrogen

bonds. Nevertheless, they rightly align the molecules long enough as to assist ligase in performing its task.

Sticky-end overhangs produced by the majority of the restriction enzymes consist of a palindrome of two or four nucleotides. In theory, only fragments containing complementary overhangs can be connected to one another by ligase. In practice, illegitimate connections are a common occurrence. Due to the low affinity between such short single strand overhangs and consequently, due to their unstable nature, legitimate ligation is an inefficient process yielding a low amount of desired product. Furthermore, the palindromic nature of the sticky ends always results in undesired by-products such as "head-to-head" connections between identical molecules which further reduces the amount of the desired product.

Connecting DNA fragments with non-matching ends can be carried out by either blunting the ends by special enzymes or by adding very short, artificial DNA molecules called "linkers". These molecules are specifically designed to have an overhang that would match one fragment on one of their sides and another overhang, on the other side, that would match the other fragment. This however further complicates the construction process, reduces the yield, increases the percentage of wrong constructs and sometimes adds undesired foreign sequences.

Because of the inefficiency of cloning restriction enzyme fragments, connecting more than two fragments at once is avoided if possible, and the building of DNA constructs is done one step at a time. Each step consists of several stages: first, desired DNA fragments are cut by restriction enzymes from larger molecules. Next, two DNA fragments are ligated to each other. Since the amount of the desired fragment is low, it has to be amplified, usually by transforming cells of choice, such as, for example, bacterial cells. In order to do so, the product has to be circular DNA and

has to contain certain components that will allow its amplification in bacteria. The third stage is therefore transformation of the DNA product into bacterial cells. Because of the high background of undesired product, a fourth, verification, stage has to be carried out. In this stage, the DNA from various bacterial clones is purified and tested in order to distinguish between the desired product and all the others. Only afterwards can one proceed to the next step. The construction of a complex DNA molecule requires numerous such steps. In terms of time, the construction of sophisticated molecules may take anywhere between several weeks to several months. Sometimes the completion of a construct is not achieved at all.

Although the affinity between complementary overhangs of two or four nucleotides is low, both the affinity between complementary overhangs and the stability of the hydrogen bonds, once formed, greatly increase when the overhangs are longer. One way to observe and quantitate the affinity is by mixing two distinct fragments with complementary overhangs and then separating them by Agarose Gel Electrophoresis: each of the fragments migrates on the gel according to its length. Fused fragments migrate as one fragment which is equal in length to the sum of both original fragments. The relative density of the DNA in the three different locations indicates the ratio between bound and unbound fragments.

One prior art method uses overhangs that are created by adding nucleotides at the 3' end of a DNA strand in a template-independent fashion (Roychoudhury, R. Gene Amplif Anal. 2:41-83, 1981). The enzyme used in this method, Terminal Transferase, will incorporate nucleotides at the end of a double-stranded DNA fragment, thus creating a single-stranded tail. Since the enzyme uses the nucleotides randomly, the only way to ensure that the single-stranded tail will be complementary to a corresponding overhang created on a second DNA molecule is to provide

only one of the four nucleotides. The overhangs created with this method must therefore be homopolymeric, so that only four types of overhangs can be used, corresponding to the residues dA, dC, dG, or dT. Since the overhangs created on both termini of a DNA fragment will be identical, cloning with this method is directionless. Furthermore, the length of the overhangs cannot essentially be controlled. Finally, the method necessarily introduces an unwanted stretch of nucleotides into the final construct, the length of which cannot be determined exactly, making the method unsuitable for the purpose of cloning into expression vectors where the reading frame must be preserved.

There is no doubt that the affinity between complementary overhangs 12 nucleotides long is greater than that of overhangs of 4 nucleotides. Various cloning kits sold in the market are based on the fact that the hydrogen bonds between overhangs 12 nucleotides long are stable enough to make the addition of ligase prior to transformation into bacteria unnecessary. The hydrogen bound fragments remain attached to one another during the transformation procedure and then become covalently bound by the bacterial ligation machinery. In fact, in a number of recent issued U.S. patents and a published PCT patent application: U.S. 5,137,814; U.S. 5,229,283; U.S. 5,334,515; and WO/18175 (PCT/US93/01965), all assigned to Life Technologies, Inc., U.S., there have been described methods for inserting DNA fragments of choice into a DNA vector of choice by way of generation of such 12 nucleotide long complementary overhangs, as well as ways of altering the nucleotide sequence of such DNA fragments at the time of their preparation and insertion into the vectors.

In Kaluz et al.(1992), Nucleic Acids Research, Vol. 20, No. 16, pp. 4369-4370, there has been described the use of the enzyme Exonuclease III (hereinafter "Exo III") for the purpose of directional cloning of PCR products. Exo III is an exodeoxyribonuclease that digests double strand

DNA from the 3' end, releasing 5' phosphomononucleotides. The above publication is also incorporated herein in its entirety by reference for all matters concerning the basic conditions in which Exo III is used. It should, however, be noted that in this publication there is described the synthesis of a single polymerase chain reaction (PCR)- generated product using two different primers, which was then digested with Exo III to yield different single-stranded 5' overhangs at each end of the PCR product. This PCR product with 5' overhangs was then inserted into a bacterial vector that was cleaved with two different restriction endonucleases to provide short single-stranded overhangs complementary to the 5' overhangs of the PCR product to enable a directional joining, facilitated by the enzyme ligase, between the PCR product and the vector. Overdigestion by exonuclease leads to single stranded gaps. The gaps are filled-in *in vivo* by bacterial DNA repair enzymes once the vector was introduced into bacterial cells. Hence, this publication does not disclose the joining of two or more PCR products to each other in a predetermined order to produce a desired product composed of such directionally joined PCR products. Moreover, the joining between the PCR product and the vector according to the above publication is at first by interaction between the short (2-4 nucleotides only) overhangs on the vector and the complementary short portion at each of the termini of the exonuclease-digested PCR product. It is now known that such interactions, namely, hydrogen bonding between short (even up to 12 nucleotides) single-stranded DNA stretches is stable but usually results in a low yield of desired product. Thus, the method in the above publication is not more effective than ligation of restriction-cleaved DNA fragments.

This above published method therefore does not significantly overcome the drawbacks of the above-mentioned previous methods for the joining of restriction endonuclease-generated DNA fragments as far as the initial connections or interactions between the short "sticky ends" is concerned. Rather, this published method serves primarily to overcome other

drawbacks of the joining of restriction enzyme fragments, namely, it allows one to generate a PCR fragment of predetermined sequence from primers of choice, the sequence of the primers being chosen so that their terminal ends, which will be the terminal ends of the PCR product, are such that upon reaction with exonuclease, there will be generated 5' overhangs at the termini of the PCR product which will be complementary to the overhangs generated by restriction enzymes on the molecule (the vector) that it is desired to join to the PCR product.

However, even this improvement over previous methods to join restriction enzyme-generated fragments still has another major drawback. Namely, the PCR product must be designed in such a way that its 5' terminal ends generated by the Exo III digestion are complementary to restriction enzyme-generated sticky ends to be generated on the vector. Hence, by this procedure, the junctions between the PCR product and the vector are still limited to restriction sites, with all the intrinsic problems thereof.

The use of overhangs longer than 4 nucleotides for the purpose of fragment cloning is also described in several other publications. Kuijper et al. (Gene 112, p. 147-155, 1992) and Aslanidis et al. (PCR Methods Appl. 4:172-177, 1994), describe a cloning method wherein T4 polymerase is used together with a predetermined dNTP to generate overhangs of a certain length in PCR products. This method requires a specific sequence to be present in the PCR primer. Hsiao et al. (Nucleic Acids Res. 21, p. 5528-5529, 1993) and Yang et al. (Nucleic Acids Res. 21, 1889-1893, 1993 and US 5,580,759) disclose generation of overhangs by the exonucleolytic activity of Exonuclease III (exonuclease) or of T4 polymerase. Overhangs of 12 (Aslanidis), 8 (Yang) and 10-14 (Hsiao) nucleotides are disclosed.

Using such complementary overhangs of only 12 or 14 nucleotides in length represents a further drawback of the above earlier methods: The efficiency of joining between two fragments in such cases is not high

enough, as shown in a copending application of the same applicant herein, filed together with the present application and identified as Attorney Docket 4191/96, (Israeli Patent Application No. 120339) . Hence when three or more of such fragments are desired to be joined, these methods do not provide a useful yield of the desired products. Thus, using up to 14 nucleotide overhangs with the prior art methods, it is likely that when it is desired to join three or more DNA molecules together, this would have to be done in a step-by-step process in which, at first, two molecules would be joined, the so-joined molecule then isolated and purified, and afterwards, the third fragment would be added thereto, and so on for any additional fragments to be joined. As a result, these prior art methods are also time-consuming and require a significant input of resources.

Accordingly, one of the aims of the present invention is to provide a method by which two or more fragments may be joined together in a specific predetermined directional manner, in which the joining step is essentially a single step and in which the junction sites between the various fragments are seamless, namely, do not contain any linker DNA segments to provide a fully in-phase joining of one fragment to the next.

In the above copending application, there is described a method for joining DNA fragments in an efficient manner, by way of a rapid procedure, and this by providing long terminal overhangs of at least 15 nucleotides on each of the fragments to be joined.

In another copending application of the same applicant herein, filed together with the present application and identified as Attorney Docket 4149/96, (Israeli Patent Application No. 120337) there is described a method for "seamless" joining of DNA fragments in which the junction sites do not contain any linker DNA segments to provide a fully in-phase joining of one fragment to the next. This method utilizes specific primers for the PCR-generation of the DNA fragments which contain the

exo-sample nucleotide dU situated within the primer, and within the subsequent PCR-generated DNA fragment at a position that following the reaction to remove this dU residue and the terminal oligonucleotide connected thereto, the DNA fragments will have 3' overhangs of a length of 15 or more nucleotides of a specific, predetermined sequence. The DNA fragments are then joined via these 3' overhangs whereby only the fragments to be joined together have complementary overhangs, and this only at the specific ends of the fragments to be joined. Such a method thus provides for an essentially one-step joining reaction in which all the individually generated DNA fragments are mixed together, treated to yield the 3'-overhangs and at the same time to join together to yield the production in which the fragments are joined in a specific, predetermined directional manner. As such, this method represents a rapid and efficient procedure to join 2 or more PCR-generated DNA fragments together and overcomes the drawbacks of the above-noted prior art.

It is another purpose of the present invention to provide a method for joining three or more DNA fragments in an efficient manner, and at the same time ensuring that there is only complementarity between the terminal overhangs of any two fragments at the specific ends of the fragments which are to be joined together, ensuring that only the predetermined desired order of joining between the fragments is achieved. In contrast to the above-noted copending applications of the same applicant herein, the present invention utilizes Exo III, or a functional equivalent thereof (hereinafter "exonuclease"), to generate specific predetermined 5' overhangs on the PCR-generated DNA fragments to be joined together in a specific directional manner. Further, unlike the inventions according to the above copending applications, the DNA fragments utilized in the present invention are not necessarily PCR products.

Elaboration of the above aims and aspects of the present invention, as well as other aims and aspects of the present invention, will be readily detailed in or will be apparent from the foregoing disclosure.

It should be noted that all of the various terms, procedures and the like, used herein throughout, unless otherwise indicated, are all well known terms in the art, known by all of average skill in the art. Thus, for example, terms such as nucleotides, primers, PCR, and the like, are readily known to all of skill in the art and are well defined in all standard texts and publications, for example, the above-noted Sambrook et al., as well. The above-noted U.S. patents and PCT application, and the publication by Kaluz et al., incorporated herein by reference, are less well known.

Summary of the Invention

In accordance with the present invention, there is provided a method for the simultaneous multi-DNA fragment assembly of two or more double-stranded DNA fragments, particularly fragments produced by primer extension reaction and in particular the polymerase chain reaction (PCR) or by restriction cleavage, or the like method, comprising the steps of:

(a) providing for each DNA fragment to be joined to a second DNA fragment and optionally to a third DNA fragment two terminal portions, the first of said two terminal portions, located at one end, being complementary to one of the termini of the second fragment, and the second of said two terminal portions, at the other end, being complementary to one of the termini of the third fragment, the complementary regions being at least 15 nucleotides in length and being designed to be unique so that in a given reaction one such region, at the terminus of a given fragment, is complementary only to the specific terminus on another fragment which it is supposed to join;

(b) carrying out in any suitable order the steps of:

(1) mixing all of the DNA fragments of in a single reaction vessel, and

(2) I. adding thereto the enzyme Exonuclease III, or a functional equivalent thereof (exonuclease), under conditions and for a period of time suitable to provide for the exonuclease digestion which will expose complementary overhangs of at least 15 nucleotides at the ends of said DNA fragments;

II. stopping the exonuclease reaction of (b) by denaturing or inactivating said exonuclease, once enough nucleotides have been removed from the ends of the strands of said DNA fragments to expose said complementary overhangs,

(c) subjecting the exonuclease-digested fragments to conditions suitable to provide for the specific joining between each DNA fragment via the complementary overhangs, and

(d) filling in gaps by adding a DNA polymerase activity and deoxytriphosphate nucleotides.

Of course, if two fragments are to be joined to a circular construct, then the above termini of said second and third DNA fragments are termini of the same fragment. Furthermore, it will be readily appreciated by all of skill in the art that it is possible, by the above method of the invention, to join a fragment at one end only, i.e., it is not necessary to join both ends of any or all of the above fragments.

By one embodiment of the above method of the invention, there is provided a method wherein the DNA fragments to be joined together are obtained by restriction cleavage of DNA molecules containing said fragments, and wherein the restriction enzymes used to provide said DNA fragments are chosen so that each fragment will have the desired first and second terminal portions which are complementary to only one terminal portion of one other fragment. Thus, by this embodiment of the invention, it will be readily appreciated that when it is desirable to combine DNA fragments

which have homology to each other, but which originate from different sources, these fragments may be combined into a new construct via joining at these homologous portions. This joining, in accordance with the present invention, as detailed hereinbelow, will be by first generating long overhangs on each fragment to be joined by exonuclease digestion of the fragments (restriction fragments) and by virtue of the fact that there is homology between the fragments, there will be a complementary interaction between the overhangs on each such exonuclease-digested fragment, enabling the joining between two such fragments. Accordingly, this embodiment of the present invention is unlike previously described procedures of the art, wherein the joining between fragments is only by the overhangs corresponding to a particular restriction site, whose overhangs are generally not longer than 4 nucleotides, whereas in accordance with the present invention, it is required that the overhangs are at least 15 nucleotides long.

In accordance with another embodiment of the present invention, there is provided a method wherein at least some of the DNA fragments to be joined together are produced by the polymerase chain reaction (PCR). The complementary regions, at the ends of the various fragments are produced as follows:

The primers which are utilized to produce a given fragment are made of two portions; 3' portions which are complementary, as in regular PCR, to the termini of the fragment which is to be amplified, and 5' portions which are complementary to the termini of the fragments which are to be joined to the given fragment. The PCR fragments that are thus amplified contain the required terminal complementary regions.

This embodiment of the present invention, as set forth in detail hereinbelow in the Examples, is a particularly versatile way in which to join any desired fragments of DNA to each other in a specific directional fashion, and is especially useful when the fragments themselves do not

have any homology to each other. Further, when fragments are joined by this method the joining will be seamless, because there is no need to add undesired sequences.

According to yet another embodiment of the above method of the invention, there is provided a method wherein the DNA fragments to be joined are a mixture of DNA fragments, some being provided by restriction cleavage and some being provided by PCR production, all of said fragments having the desired first and second terminal portions which are complementary to only one terminal portion of one other fragment. This embodiment of the present invention is particularly useful wherein it is desired to join a particular restriction fragment of choice which is readily obtainable by restriction cleavage to another DNA fragment of choice, but which does not have the matching restriction sites. Accordingly, by selecting the desired primer sequence which will contain a portion of the sequence of the restriction-generated fragment at one of its termini and the sequence of a portion of the second fragment at its other teminus, it is possible to generate a PCR fragment which will have a terminal end which is homologous to one of the terminal ends of the restriction-generated fragment, and in this way, by the method of the present invention, the two fragments may be joined together.

With respect of any one or all of the above embodiments of the present invention, there is provided a method wherein the joining of the exonuclease-digested DNA fragments *in vitro* comprises:

(a) heating the reaction mixture after stopping the exonuclease reaction to about 75°C and incubating said mixture for a period of time sufficient to disconnect illegitimate connections between the fragments at said temperature;

(b) slowly cooling the heated mixture of (a) to promote the specific joining of complementary overhangs; and

(c) adding to the cooled mixture of (b) at about 37°C the enzyme T7 DNA polymerase, or T4 DNA polymerase, or a functional equivalent thereof, as well as dNTPs, and optionally also adding the enzyme T4 DNA ligase, under suitable conditions to facilitate filling in and ligation of the strands of the joined fragments.

Alternatively, the above steps (a) and (b) may be replaced by incubation of the fragments at 37°C or at a temperature suitable to allow correct hybridization of the overhangs.

It should therefore be noted that the actual joining of the exonuclease-digested fragments may be partly facilitated *in vitro*. The use of Ligase in the final step of the method of the invention to covalently join the DNA strands of the combined fragments, is optional and this in view of the fact that using complementary overhangs of at least 15 nucleotides and filling in the gaps provides a sufficiently strong interaction between the joined fragments so as not to disconnect during transformation and/or transfection of host cells with the DNA construct according to the invention.

In accordance with any one of the embodiments of the present invention, as set forth hereinabove and as detailed hereinbelow, the DNA fragments to be joined are selected from two or more DNA fragments having regulative functions such as promoters, enhancers, terminators, ribosome binding sites, and the like, and DNA fragments encoding proteins such as enzymes (such as citrate synthases, poyketide synthases, succinyl-CoA-synthases), cytokines, hormones, and the like. Accordingly, by the method of the present invention, it is readily possible to, for example, attach a new promoter of choice to a new DNA fragment encoding a protein of choice to provide for a new construct in which the protein will be under the control of the promoter. The number of such combinations is essentially endless. Moreover, the present invention has the advantage of joining several such fragments simultaneously.

Furthermore, by the method of the present invention, it is also possible to generate site-specific mutations in any one or more of the fragments to be joined at the time that these fragments are prepared for joining. For example, if the fragments are to be produced by the PCR procedure prior to their joining, the primers for this PCR procedure may be synthesized in such a way as to have a specific site mutation anywhere in the primer sequence or even more than one site-specific mutation, such that the resulting PCR-generated fragment will contain one or more site-directed mutations in its sequence. Accordingly, another embodiment of the method of the present invention is one in which one or more of said DNA fragments to be joined is a mutant fragment having been subjected to site-directed mutagenesis during its preparation, as pointed up above. Accordingly, the resulting DNA construct will be one containing one or more site-directed mutations. This embodiment of the present invention is particularly useful when it is desired, for example, to join a particular regulatory DNA molecule to a particular protein, but at the same time, to alter the activity of either the regulatory element (for example, to have a promoter having a higher or lower than normal activity) or the protein to be expressed (for example, to have a protein with altered acitivty). It is also possible to introduce mutations by the use of PCR conditions that result in a high rate of mutations in the amplified fragments.

In accordance with the present invention, the exonuclease used is typically Exonuclease III or T4 DNA polymerase. However, other 3' to 5' exonucleases may be used. Also 5' to 3' exonucleases are useful in the practice of the present invention.

In accordance with the present invention, there is also provided an assembled DNA construct whenever prepared by any one of the above-mentioned methods of the present invention or any one of the

embodiments thereof, and wherein said assembled DNA construct has been assembled from the joining together of two or more DNA fragments.

Illustrative and non-limitative examples of the above assembled DNA construct of the invention are:

a DNA construct having been assembled by the joining together of three DNA fragments;

a DNA construct having been assembled by the joining together of four DNA fragments;

a DNA construct having been assembled by the joining together of five DNA fragments;

a DNA construct according to any of the above-mentioned constructs in which the construct is in the form of a linear DNA molecule; and

a DNA construct according to any of the above-mentioned constructs in which the construct is in the form of a closed circular DNA molecule.

The present inveniton also provides a DNA fragment comprising an overhang of at least 15 nucleotides or an end portion suitable to be converted into such an overhang. Further, the invention provides said DNA fragment, for use in the above method.

Other embodiments and aspects of the present invention will be apparent from the following detailed description of the invention.

Brief Description of the Drawings

- Fig. 1 (A-E) is a schematic diagram illustrating the joining between two, out of two or more, joining fragments in accordance with the method of the present invention, as is detailed in the Examples, and wherein the regular lines depict the single strands of the double-stranded DNA fragments, the broken lines denote those 3' portions of the DNA strands which are digested by the action of exonucleases on each of the DNA fragments, the dotted lines indicate gaps in the junction region

between the two DNA fragments which are filled in by a DNA polymerase, such as T7 DNA polymerase, and p indicates the phosphorylated 5' ends of the strands of the double-stranded DNA fragments (a phosphorylated 5' end is required for the ligation reaction whenever ligase is used);

- Fig. 2 is a schematic diagram of a plasmid molecule prepared in accordance with the present invention from 3, 4 or 5 separate DNA fragments, as detailed in the Examples, wherein the portion of the plasmid depicted by the open lines represents the Tet^r gene which may be synthesized as one fragment using primers SEQ. ID NO. 5 (also designated 31160) and SEQ. ID NO. 6 (also designated 30397), or as two fragments, Tet^r A and Tet^rB, in which the Tet^rA fragment is synthesized from primers SEQ. ID NO. 5 and SEQ. ID NO. 10 (also designated 27336) and fragment Tet^rB is synthesized from primers SEQ. ID NO. 6 and SEQ. ID NO. 9 (also designated 25586), in all instances the Tet^r fragments or the Tet^rA and the Tet^rB fragments being synthesized using plasmid pBR322 as template; the dark bar depicting the Amp^r + ColE1-ORI fragment which is synthesized from primers SEQ. ID NO. 4 (also designated 3884) and SEQ. ID NO. 3 (also designated 4142) using plasmid pBR322 as template; the dashed bar depicting the Cm^r fragment which may be synthesized as a single fragment from primers SEQ. ID NO. 8 (also designated 4143) and SEQ. ID NO. 7 (also designated 3595), or as two separate subfragments Cm^rA and Cm^rB, which are synthesized from primers SEQ. ID NO. 7 and SEQ. ID NO. 1 (also designated 27337), and primers SEQ. ID NO. 8 and SEQ. ID NO. 2 (also designated 25587) respectively, in all cases the Cm^r fragment or subfragments being synthesized using plasmid pACYC184 as template, and wherein the longer vertical lines between the aforesaid fragments or subfragments denoting the junction point between the fragments, and wherein above the number corresponding to each of the aforesaid primers there is an arrow depicting the direction of synthesis from the primer, the arrowhead being the 3' end of the primer. The length of the various fragments is also indicated.

Fig. 3 shows several constructs created, as shown in example 4, by joining 3 (Fig. 3A), 4, (Fig. 3B), or 5 (Fig. 3C) fragments. The general method of designing the primers is as detailed for Fig. 2 above.

Detailed Description of the Invention

The present invention concerns an improved method for combining two or more DNA fragments together into a multi-DNA fragment assembly, by which method the fragments are joined in an essentially single-step joining reaction, in a desired, predetermined order and in a seamless fashion, namely, no linker DNA is inserted between the joined fragments.

The DNA fragments to be joined together are produced by PCR. This is achieved, in accordance with this embodiment of the present invention, by synthesizing specific pairs of primers for the PCR production of each fragment. Each primer is synthesized by standard, automated single-stranded (oligonucleotide) DNA synthesis and has two parts, the first part being complementary to one of the terminal portions of the fragment to be produced, and the other part being complementary to the terminal portion of the strand of another fragment to be specifically joined to this first fragment. Thus, the primers, in accordance with the present invention, essentially correspond to the desired predetermined junction region at those ends of the two fragments to be joined. In this way, for each fragment generated, a unique pair of primers is used.

According to the present invention, the above pairs of primers, when employed in the PCR procedure, yield DNA fragments having the desired specific complementary terminal sequences. To join these fragments together, it is necessary to generate complementary single-stranded overhangs at the respective termini of the fragments to be joined. These are generated by the use of exonuclease under controlled conditions (as detailed hereinbelow), whereby the exonuclease digests each of the two strands of each of the DNA fragments starting at the 3' terminus of each

strand to yield DNA fragments having 5' overhangs. The extent of digestion with exonuclease is controllable, and hence the length of the 5' overhangs may be controlled relatively accurately. By controlling both the temperature of the exonuclease reaction and the incubation time, it is possible to fairly accurately control the extent of the degradation, and hence, the length of the resulting overhangs. The exonuclease reaction is also easy to stop by, for example, adding EDTA and heating to about 72°C, or by performing a phenol extraction, at the end of the desired duration of the reaction.

When using exonuclease in accordance with the present invention, the PCR-generated DNA fragments to be joined together can be readily prepared having at least 15 nucleotide-long 5' overhangs, and preferably 5' overhangs of 20 nucleotides or more.

In accordance with the present invention, following exonuclease degradation, long 5' overhangs are formed. As the exonuclease digestion of the fragments is not perfectly uniform, it often occurs that some strands of the DNA will be digested more than others, such that for a particular junction region one 5' overhang may be longer than its complementary one, with the result that a gap of one or more nucleotides may form in one or even both strands at the junction region. In such instances, the junction will be maintained by virtue of the hydrogen bonding between the two strands, which is relatively strong and stable when the interacting overhangs are at least 15 nucleotides in length. According to the invention, the gaps are repaired *in vitro,* by DNA repair enzymes, for example, using T7 DNA polymerase. Optionally, Ligase (usually T4 DNA Ligase) may then be added. When T7 DNA polymerase is used to fill the gaps *in vitro*, it may be added with the ligase to ensure that in a still essentially one-step joining reaction, the fragments are covalently linked to each other to provide the desired construct.

It should also be noted that the joining of fragments according to the present invention may be carried out using DNA fragments generated by restriction enzymes, but in this case, care must be taken to ensure that each fragment has the sequence of choice, as well as the possibility of yielding the necessary respective 15 nucleotide or longer 5' complementary overhangs.

According to one embodiment of the invention, when the DNA fragments to be joined are PCR fragments, then the primers should be phosphorylated to add a phosphate at their 5' termini to facilitate the ligation step mediated by ligase.

All of the above aspects of the present invention, including the primer synthesis, PCR synthesis, exonuclease reaction, T7 DNA polymerase, T4 DNA polymerase and ligase reaction, are detailed hereinbelow.

In accordance with the present invention, as mentioned above, overhangs of at least 15 nucleotides long are generated to ensure stable and efficient joining between the various fragments. In fact, in a series of experiments in which overhangs of only 12 nucleotides were employed as disclosed in example 4 below and in the above-mentioned copending application (identified as Attoreny Docket 4191), it was found that only low efficiency joining between two fragments could be achieved, and no joining between three or more fragments into a single construct could be achieved. Hence, in accordance with the present invention, it has been found that such overhangs must be at least 15 nucleotides long to ensure the joining of two or more fragments together in an efficient manner.

In accordance with the present invention, it is of course also possible to generate one or more site-specific mutations within the one or more fragments to be joined together and this by standard procedures, in which the various above-noted primers, when generated, are generated having

specific nucleotide substitutions, deletions or additions at a selected site or sites within the primer or primers. Subsequently, the fragment generated from such a primer will have the pre-selected site-specific mutation. Likewise, it is also possible to generate regions of either high mutation rates or very low mutation rates by amplifying fragments using mutagenic PCR protocols or high-fidelity enzymes, respectively.

As regards the various DNA fragments to be joined in accordance with the method of the present invention, these fragments may encode any DNA molecule of choice. For example, the fragments may encode various regulatory molecules such as, for example, promoters, enhancers, terminators or the like. The fragments may also encode various proteins having various biological activity of pharmaceutical or veterinary importance, for example, various metabolic enzymes, hormones, cytokines, and the like. Hence, it is possible to generate, in accordance with the present invention, new chimeric promoters having improved activity and this by joining two fragments together encoding parts of promoters from various sources. Likewise, it is possible to generate a wide range of chimeric structural and regulatory proteins, for example, chimeric cytokine molecules, receptors, enzymes and the like, of improved or desired biological activity, by combining fragments encoding different domains of such molecules from different sources. Likewise, a multi-fragment assembly may be devised in which a new desired promoter is directly connected to a new desired DNA molecule encoding a biologically active protein in a single new construct for the purposes of enhanced expression of this new desired protein, once the construct is used to transfect/transform suitable cells of any organism of choice, for example, prokaryotic or eukaryotic cells such as bacterial or yeast cells, respectively, or mammalian, insect or any other eukaryotic cells. Likewise, such a multifragment assembly can also be in the form of a modified bacterial or animal virus carrying one or more genes of choice for

the purposes of infecting prokaryotic or eukaryotic cells of choice, and thereby introducing into these cells the gene(s) of choice.

In accordance with the present invention, the PCR procedure is essentially the well known, now standard, procedure, which may be augmented by using recently described new high-fidelity DNA Pol enzymes, as well as newly developed, improved automated machinery for this purpose. Hence, any PCR procedure and reagents for use therewith may be utilized in accordance with the present invention to generate the very specific fragments which are to be combined together.

In accordance with the present invention, one may readily prepare a DNA construct of choice, namely, a "custom-made" DNA construct in which any desired DNA fragment encoding any desired structural or regulatory function, can be joined in a seamless fashion to other such DNA fragments. As the procedure in accordance with the present invention is both rapid, simple to perform, and allows for the simultaneous joining of several fragments, it is therefore possible to prepare any DNA construct of choice, be it a linear DNA molecule for insertion into cells directly by known techniques, or a circular DNA molecule to be used as a vector for transfecting/transforming cells of choice, or a linear construct for insertion into another vector of choice, and any other such purpose readily apparent to any of skill in the art. The list of possible constructs which may be prepared in accordance with the present invention is essentially endless.

The present invention will now be described in more detail in the following non-limiting examples and the accompanying drawings:

The General Procedure

According to one embodiment of the present invention, the fragments which are to be attached to each other in a directional fashion are preferably prepared by utilizing the well-established Polymerase Chain

Reaction (PCR) procedure, this being a standard procedure of the art. This provides for suitable amounts of the fragments and is especially preferred when the fragments to be used are originally obtained or available only in small amounts, for example, from genomic DNA or DNA from cDNA libraries in which the desired fragments are present in small amounts.

In this way, the original fragments are greatly amplified and by virtue of the use of pre-selected specific primers in the PCR procedure, the 5' and 3' ends (termini) of the PCR-prepared (amplified) fragments will have the desired pre-selected sequences which will ultimately provide for the directional attachment of the fragments to yield the DNA molecule of choice, in which all of the fragments have been attached to each other in the pre-selected order.

Once obtained, the PCR-generated DNA fragments are either mixed or separately subjected to exonuclease degradation by incubating the fragments in the presence of exonuclease at a sufficiently low temperature, enabling the control of the rate of exonucleasedegradation, and for a sufficient incubation period to ensure that nucleotides are degraded from the 3' ends (when said exonuclease is a 3'-5' exonuclease) of each DNA strand of each fragment to yield 5' overhangs of at least 15 nucleotides. An example of such conditions, provided in accordance with the present invention, is an incubation period of about 10-20 minutes at 6°C with Exo III. At the end of the incubation period, the Exo III degradation reaction is stopped, usually by performing a phenol extraction or by adding EDTA and heating the reaction mixture to about 72-75°C. A phenol extraction is preferred, even though it adds another step to the reaction, albeit a simple and short step, because the Exo III is completely denatured by the phenol, and at the same time, the DNA fragments are obtained in a purified form after the phenol extraction, ensuring that no undesired subsequent Exo III degradations will occur at the stage when it

is desired to join the fragments. Alternatively, any other method to stop the reaction can be used, for example by using commercially available kits (see example 4 below).

Following the phenol extraction (or as part of the EDTA-induced cessation of the Exo III reaction), the mixture containing DNA fragments with exposed 5' overhangs is then heated to 75°C for about 5 minutes and is then cooled very slowly, this heating and cooling representing the initiation of the desired joining reaction. When the temperature reaches 37°C, a DNA polymerase is added to the DNA fragments mixture (essentially joined fragments via complementary hydrogen bond interaction) together with dNTPs and with or without ATP and ligase, and incubated at 37°C for about 2 hours, to facilitate the filling-in of any gaps in the junction regions between the various joined DNA fragments to yield a fully covalently joined product containing all of the fragments joined in the predetermined order.

It should be noted that, with respect to the above reaction conditions: (i) at 6°C the 3' ends of the DNA fragments are sufficiently slowly degraded by excess amount of Exo III so as to be controllable to yield 5' single-stranded overhangs at both termini of each fragment, and at this temperature, the period of incubation time with Exo III is the main parameter determining the length of the 5' overhangs on each fragment, rendering the reaction easy to control by simply checking the time of incubation and stopping the reaction when the desired period has elapsed; (ii) The heating of the Exo III-degraded fragments is primarily to cause disconnection of any mis-hybridizations (or joining) between the various fragments which may have occurred during the Exo III reaction at 6°C. The subsequent very slow cooling maximizes the legitimate or desired hybridizations or connections between the various fragments via the complementary 5' overhangs (alternatively, the hybridization can be done at 37°C or at any temperature temperature that allows joining of

complementary overhangs); (iii) As the Exo III degradation is not perfectly uniform, some 5' overhangs may be longer than others, such that at any junction region between two DNA fragments, which junction is maintained by the hydrogen interactions between the complementary overhangs, there may arise gaps of one or more nucleotides at one or both sides (on one or both DNA strands) of each junction region. Thus, a DNA polymerase, such as T7 DNA polymerase or T4 DNA polymerase is used to fill in these gaps.

When T4 DNA polymerase is used as the exonuclease in the reaction, a different incubation time and temperature are used, namely, 37°C for 2 minutes. By the above preferred procedure, in accordance with the present invention, two or more DNA fragments may be linked, preferably three, four, five or more DNA fragments may be linked in a specific directional manner.

For the purpose of simplicity, it should be noted that in the above general procedure, when, for example, it is desired to connect five PCR fragments together to form a linear molecule, then, the specific primers used in the PCR step are designed in such a way that the ends that are to be connected to each other are complementary (i.e., have perfectly complementary 5' overhangs, once these overhangs have been generated as noted above and below). For example, if a linear DNA construct is to be made from five fragments numbered 1-5 and for each fragment, for the purposes of illustration, we designate the sense direction to be "left to right" such that when joined fragment number 1 will be the extreme left-hand side and fragment 5 will be the extreme right-hand side of the combined molecule (or by convention, the (+) sense strand will begin with its 5' terminus at the extreme 5' end of fragment 1 and end with its 3' terminus being at the extreme end of fragment number 5; and the (-) non-sense strand will have its extreme 5' end at the extreme end of fragment 5 and its 3' end at the extreme end of fragment number 1).

Hence, to make this construct of five fragments, the right terminus of fragment 1 should be homologous to the left terminus of fragment 2, the right terminus of fragment 2 should be homologous to the left terminus of fragment 3, the right terminus of fragment 3 should be homologous to the left terminus of fragment 4, and the right terminus of fragment 4 should be homologous to the left terminus of fragment 5. With such homology, once the 5' overhangs at the ends of each fragment are generated (as noted above and below), there will thus be perfect complementarity between the 5' overhang at the right terminus of fragment 1 and the 5' overhang at the left terminus of fragment 2 to provide for a specific directional joining of these two fragments by complementary interaction or joining between the two 5' overhangs, and likewise for the joining of the above-mentioned other termini of the various fragments to be joined. In such a construct, therefore, the left terminus of fragment 1 and the right terminus of fragment 5, i.e., the extreme ends of the molecule, should not have any homology to each other or to any of the other left or right termini of all of the various fragments, in this way ensuring that the so-produced molecule will be linear and will not be capable of circularization or undergoing any other inter-fragment interactions which would disturb the 1-2-3-4-5 desired configuration to be formed.

However, when it is desired to make such a molecule which is circular, then of course the right terminus of fragment 5 should be homologous to the left terminus of fragment 1, thereby ensuring that circularization occurs only by connection between fragment 5 and fragment 1. As also arises from the above-mentioned, all of the left and right termini of each of the fragments which are not designed to be connected must be non-homologous, this condition being relatively easy to meet in view of the standard technology available for the automated synthesis of primers, the sequence of which is easy to determine.

Thus, it is apparent from the above general procedure that the method of the present invention is essentially a single-step procedure in which all of the reaction components are present in a single reaction vessel in which all of the various reactions and treatments are carried out, thereby greatly simplifying the overall process and providing an end-product that is essentially only the desired product which can be readily obtained and further utilized.

It should of course be noted that the initial PCR amplification of the fragments which it is desired to combine should be carried out in separate reaction vessels for each specific fragment to ensure the fidelity of the PCR products and this in view of the fact, as noted above, that for any two fragments to be combined, there is a necessity for homology at those ends of the two fragments which will be connected together.

However, this requirement for separate PCR amplifications of the selected sequences is now also a simple laboratory procedure in view of the widely available automated apparatuses for carrying out PCR procedures, which can handle a large number of separate samples at the same time. For example, the production of the desired PCR primers in accordance with the present invention, may be carried out by automated machinery which allows for the generation of large numbers of primers of high fidelity and purity simultaneously in a very short period of time (for example, there are machines which can simultaneously produce 96 different primers at a cost of only about 10-30 cents per base per primer). Hence, it is possible to readily produce any desired primer having the desired predetermined sequence. Further, by standard methods of the art, each such synthesized primer may also be readily 5' phosphorylated to provide for 5' fragments, which can then be easily ligated following the filling-in step.

A schematic representation of the above-mentioned general procedure is set forth in Fig. 1, wherein in part A there is shown schematically two

double-stranded DNA fragments (for the purposes of simplicity of illustration only, as it should be understood that the procedure is effective for joining 2, 3, 4, 5 or more such fragments together), "fragment 1" and "fragment 2", which are to be joined together. For illustration purposes, the junction between fragment 1 and fragment 2 will be between the righthand end of fragment 1 and the lefthand end of fragment 2, and hence the fragments are synthesized by the PCR procedure using primers that will, as noted above, render the righthand end of fragment 1 to be homologous to the lefthand end of fragment 2. In part B, there is shown schematically the exonuclease degradation of fragments 1 and 2, which generates 5' overhangs on each end of each fragment. As the exonuclease degrades in a 3'-5' direction, both strands of each fragment will be degraded starting from their 3' ends, this being illustrated by the interrupted line in part B of Fig. 1, this interrupted line actually representing the extent of nucleotides removed from each 3' end of each fragment. As noted above, the extent of exonuclease degradation will be determined by the length of incubation of the fragments or by the temperature of the reaction with exonuclease. At the end of the exonuclease reaction, each fragment will therefore have both a lefthand and righthand 5' overhang, as illustrated in part B of Fig. 1, and wherein, as noted above, by design of the primers overhang, the righthand (lower) 5' overhang of fragment 1 will be complementary to the lefthand (upper) 5' overhang of fragment 2, and this in view of the fact that the righthand portion of fragment 1 is homologous to the lefthand portion of fragment 2 by design (by virtue of the predetermined primer sequence, and hence resulting fragment sequence) such that as far as complementarity goes, in these homologous regions, the lower strand of fragment 1 will be complementary to the upper strand of fragment 2 and the upper strand of fragment 1 will be complementary to the lower strand of fragment 2, but, however, as a result of the exonuclease degradation in these homologous regions of fragment 1 and fragment 2, there will only remain the lower portion of fragment 1 (the righthand 5' overhang) and the upper portion of

fragment 2 (the lefthand 5' overhang). Furthermore, in part B of Fig. 1, the solid lines represent those portions of the strands of each of the fragments which were not degraded by exonuclease.

Following the exonuclease degradation of fragments 1 and 2, the fragments are subjected to heating and very slow cooling to facilitate high-fidelity complementary interactions between the two fragments, so as to permit the joining of these fragments, as is illustrated in part C of Fig. 1 (alternatively, the annealing can be done at any temperature sufficient to allow the correct joining of the fragments), in which, in the lower portion of part C, there is shown how the righthand (lower) 5' overhang of fragment 1 becomes situated under the lefthand (upper) 5' overhang of fragment 2, and this because of the complementarity between these two 5' overhangs, the two overhangs interacting with each other and thereby forming the first major junction between the two fragments by way of hydrogen bonding. As also illustrated in part C of Fig. 1, there exist gaps at the junction region between the two fragments, these gaps being between the 3' end of the upper strand of fragment 1 and the 5' end of the upper strand of fragment 2, as well as the 5' end of the lower strand of fragment 1 and the 3' end of the lower strand of fragment 2. To repair these gaps, they are filled in by the action of T7 DNA polymerase or T4 DNA polymerase, as illustrated schematically in part D of Fig. 1. In part D of Fig. 1, the gaps that are filled in by T7 or T4 DNA polymerase are shown by the short dotted lines. At the time of filling-in the gaps with DNA polymerase, ligase may optionally also be added to the reaction mixture for the purposes of covalently joining the ends of each of the strands to each other to provide a covalently bound continuous upper strand and lower strand, this illustrated in part E of Fig. 1. Thus, following the ligation reaction, the above process has provided a fully joined molecule, in which fragment 1 has been joined to fragment 2 in a seamless fashion, and this by virtue of the complementary overhangs which constitute the junction region.

It should be noted that in the above procedure, it is preferable to allow the exonuclease digestion reaction to proceed for that period of time which will provide for at least the degradation of the complementary regions of the various fragments, these being at least 15 nucleotides and, more preferably, 20 or more nucleotides from each 3' end, and this to ensure a highly specific interaction between the complementary 5' overhangs and thereby high specificity of joining between the fragments.

Moreover, as will be detailed below, the selection of the primer sequences for the construction of each fragment to be joined are such that these sequences are derived from the sequences of the DNA fragments themselves which are to be joined together, such that there is no addition of any linker sequences, thereby ensuring that upon the joining of the fragments, this joining is in fact seamless and only the desired sequence information is incorporated into the joined molecule.

Materials and Methods for Examples 1-3

All of the fragments which were joined together, as set forth in the following examples, were first individually prepared by the PCR procedure using a commercially available PCR apparatus (Robocycler Gradient 96™, Stratagen, U.S.A.) according to the manufacturer's instructions.

The concentrations of the reagents used in all of the PCR procedures, were as follows:

Reagent	Concentration	Volume in Reaction Vessel
DNA*(see below)	0.1 µg/µl	0.5 µl
Primer 1	10 pmol/µl	10 µl
Primer 2	10 pmol/µl	10 µl
dNTP**(see below)	2.5 mM (each)	16 µl
10X Buffer	--	20 µl
H_2O	--	144 µl
TaqDNA Polymerase***(see below)	5 U/µl	0.8 µl
		200 µl - Final Volume

The temperature regime for all of the PCR procedures was as follows, in accordance with the manufacturer's instructions:

(i) 94°C for 60 sec., 40°C for 80 sec., 72°C for 240 sec., this temperature regime being carried out for 30 cycles and then followed by a final 72°C for 600 sec. After the last 72°C incubation, the reaction vessel was cooled to 6°C, in which the reaction products may be maintained for an indefinite period.

*The DNA which was used as the template in all of the following examples was either the plasmid pBR322 or the plasmid pACYC184, both commercially available and for both the full sequence and restriction maps being available. This will be described in more detail in Example 1 below.

**dNTP is a mixture of dATP, dTTP, dCTP and dGTP, all obtained from commercial suppliers and used in accordance with the manufacturer's instructions (Boehringer-Mannheim, Germany).

***The Taq DNA polymerase was also obtained from a commercial supplier and used in accordance with the manufacturer's instructions (Boehringer-Mannheim, Germany).

Once the various DNA fragments were produced by the PCR procedure, each PCR fragment being produced separately, the various PCR fragments were then mixed together in a single reaction vessel and subjected to the conditions necessary to facilitate the joining of the fragments. This joining procedure is an essentially one-step procedure in which the mixture of the various PCR fragments are digested by the Exo III enzyme under incubation conditions which will yield, as noted above, 5' overhangs at each end of the fragment that will be at least 15 nucleotides in length and preferably about 20 or more nucleotides in length. After the predetermined desired period of incubation with Exo III, it is inactivated, usually by a simple phenol extraction, the resulting mixture of Exo III-digested PCR fragments obtained from the aqueous phase of the phenol extraction, then being heated and slowly cooled, as noted above. After the cooling of the mixture to about 37°C, the mixture of PCR fragments which have already essentially joined together in a specific directional manner by way of hydrogen bonding between the various specific complementary 5' overhangs at those ends of the fragments to be joined, the T7 DNA polymerase reaction and ligase reaction are initiated by addition of these enzymes to provide for filling in of any gaps in any of the junction regions due to the Exo III digestion which may have rendered some 5' overhangs longer than others, and to provide for covalent ligation of the two DNA strands making up the joined molecule. Once ligated, the resulting DNA construct is then tested for the specificity of the joining, and for whether or not the joined construct has the desired expected biological activity.

The above Exo III digestion, joining reaction culminating in the T7 polymerase and ligase reactions and subsequent analysis of the resulting DNA construct was carried out according to the following procedures:

a) PCR fragments were produced from the various specific primers (see Examples 1-3 below for the specific fragments produced by PCR and the primers used for each fragment). The PCR procedure is as noted above. Each PCR fragment was prepared in a separate reaction vessel. Following PCR synthesis, each PCR fragment, separately, was subjected to agarose-gel purification using a commercial kit, namely, Bio-Rad's Prep-A-Gene™ purification kit and following the manufacturer's instructions.

b) Once purified, each PCR fragment was then quantitated by determining the DNA concentration of each fragment by standard procedures, this was performed using a commercial device, namely, Pharmacia's Gene-Quant™ RNA/DNA calculator and following the manufacturer's instructions.

c) The PCR fragments were then subjected to the Exo III digestion and subsequent joining procedure. The various PCR fragments were mixed together (0.15 pmol DNA for each fragment), in a cooled (6°C) reaction mixture of 12 µl containing: 1.2 µl 10 x TA buffer (330 mM Tris-acetate, pH 7.8; 660 mM potassium acetate, 100 mM magnesium acetate and 5 mM DTT); 0.8 µl Exo III (200 U/µl purchased from Epicentre Technologies); and sterile double distilled H_2O to make up the final volume of 12 µl. In practice, the PCR fragments were mixed into a precooled, namely, 6°C, TA buffer solution made up to 11.2 µl with the H_2O, to which was then added the 0.8 µl Exo III. Adding the Exo III last provides for better control over the Exo III reaction, which reaction is controlled by the time of incubation of the PCR fragments with the Exo III. Following the Exo III addition, the reaction mixture was then incubated at 6°C for 40 mins. (the time necessary to achieve more than 20 nucleotide degradation of each DNA strand in the 3'-5' direction under the above conditions of temperature = 6°C and concentration of Exo III).

d) The Exo III reaction was then stopped by performing a phenol extraction. This is done by adding to the above reaction mixture an equal volume = 12 µl of a 1:1 (v/v) phenol/chloroform mixture which causes denaturation of the Exo III. The aqueous phase is then separated from the above phenol/chloroform mixture, this aqueous phase containing the PCR fragments. The separated aqueous phase is then subjected to three washes with chloroform to yield a final aqueous phase having an essentially purified mixture of the Exo III-digested PCR fragments.

e) To the vessel containing these Exo III-digested PCR fragments there is then added 40 µl Mineral Oil (to prevent evaporation of the buffer and drying of the fragments) and the mixture is then heated to 75°C, at which temperature it is further incubated for one hour. After this incubation, the mixture is slowly cooled, until it reaches 37°C (the heating and cooling to provide for specific complementary interactions between complementary overhangs on the PCR fragments and to prevent non-specific interactions). This heating and cooling represents the first stage of the specific joining between the fragments, the joining by way of hydrogen bonding between complementary overhangs.

Once the above mixture has reached 37°C, it is then subjected to the final stage of the joining, including the filling in reaction, as follows:

f) To the cooled (at 37°C) mixture of now essentially joined (by hydrogen bonding of complementary overhangs) PCR fragments there is added 10 µl of the 'Synthesis Mixture', which contains:

1 µl of 20 mM ATP

4 µl of 2.5 mM dNTPs (= dATP, dTTP, dCTP and dGTP in equal amounts, concentration of each = 2.5 mM)

1 µl of 10 x TA buffer (see above for constituents)

1 µl of T7 DNA polymerase (5 U/µl, purchased from USB)

1 µl of T4 DNA ligase (10 U/µl, purchased from Epicentre Technologies)

2 µl of sterile double-distilled H_2O

Total volume: 10 µl

The above synthesis mixture has the T7 DNA polymerase and dNTP to facilitate the filling in of gaps in the junction regions between the joined fragments, as well as the ATP and the T4 ligase to covalently join the DNA strands together once the filling in of gaps has been completed.

Following the addition of the synthesis mixture, the resulting reaction mixture is then incubated for two hours at 37°C. After this incubation, the reaction mixture is then ethanol precipitated under standard conditions to finally yield a pellet of precipitated DNA which is essentially the completed DNA construct composed of the joined PCR fragments. This DNA pellet is resuspended in 5 µl sterile double-distilled H_2O and is ready for further analysis or use.

g) A 2 µl aliquot of the 5 µl H_2O-resuspended, DNA product was taken and used to transform, by way of standard electroporation techniques, DH10B *E. coli* cells (20 µl electropotent ElectroMax cells, purchased from GibcoBRL) using BioRad's *E. Coli* Pulser Apparatus (1.8 kV, 25 µF, 200 Ω, in accordance with the manufacturer's instructions). The electroporated cells were then plated on an LB agar plate containing 100 mM ampicillin. Colonies grown on this plate must all be ampicillin resistant by virtue of receiving the Amp^r gene carried in the DNA construct produced as above. Such ampicillin resistant colonies were then tested further by growing them on plates containing Tetracycline and on plates containing chloranphenicol. Those colonies capable of growing on all three plates therefore represent colonies of transfected/transformed cells which received an intact, desired DNA construct containing all three gene regions (whether constructed from 3, 4 or 5 PCR fragments).

Further specific details of each DNA construct (from 3, 4 or 5 PCR fragments) and how it was produced, are provided in the following non-limiting examples.

Preparation of a circularized plasmid by the directional connection of individual fragments constituting the plasmid

Overview of Examples 1-3

(1) The plasmid to be constructed was designed to have three or more different regions, each to be prepared separately by PCR amplification and then joined in a specific directional fashion to provide a circularized plasmid as the end-product. This required the initial preparation of specific primers, the sequence of which was to provide the basis on which the various PCR fragments, constituting the various regions of the plasmid, would be able to combine with each other to ultimately connect all the regions in the specific directional manner. The sites within the primer sequences which would provide for the interconnection between the various PCR fragments were determined irrespective of any restriction enzyme sites or any other DNA sequence features at these connection sites in order to demonstrate that, in accordance with the method of the present invention, any DNA sequence at any particular site within a DNA molecule can be utilized as a connection site by preparing the necessary specific primers to provide for this connection site.

In Fig. 2, there is shown schematically the plasmid that was designed and produced by the method of the present invention. This plasmid carries three independent antibiotic resistance genes, for resistance to ampicillin (Amp^r gene, or hereinafter Amp^r); Tetracycline (Tet^r gene, or hereinafter Tet^r); and Chloramphenicol (Cm^r gene, or hereinafter Cm^r). The plasmid also carries the ColE1 origin of replication (ColE1-ORI), which in this specific instance is situated next to the Amp^r gene, thus these two entities

constituting a single region of the plasmid. The Tet^r and Cm^r genes constitute additional separate regions. Hence, such a plasmid is capable of being replicated in a host cell to provide a number of copies of the plasmid per host cell and will endow the host cell, successfully transfected or transformed therewith, with resistance to all three types of antibiotic. Accordingly, it is also possible to readily screen for those host cells transformed by this plasmid by growing the cells in the presence of all three antibiotics and only those cells which received a functionally intact plasmid construct will be able to survive. This therefore provides for a functional analysis of the end-products of the construction procedure at the genetic level.

In order to make sure that the constructs within the transformed colonies are the correct ones, additional PCR verification tests were carried out: Pairs of primers originally used to produce two different fragments were used in a PCR test procedure in which the template DNA was the construct DNA obtained from the transformants. The PCR procedure and conditions were the same as noted above under "Materials and Methods". In this PCR test using this pair of primers, if the original fragments (within the template DNA) had connected in the correct order, then expected PCR products would be obtained, these products having an expected size. Thus, such PCR products were subjected to standard agarose gel electrophoresis and the resultant bands separated on the gel were analyzed against standard molecular weight markers to determine their approximate size and to determine whether these sizes are the expected ones.

To prepare the above plasmid, a number of constructions were made in which 3 (this example, Example 1, see below), 4 and 5 (Examples 2 and 3, respectively; see below) individual fragments were produced by PCR and then combined to yield the plasmid. Thus, when the plasmid was to be constructed from three fragments, the above-noted three regions of the

plasmid were PCR synthesized using appropriate primers so that three PCR fragments together having all three regions would be obtained. Likewise, when the plasmid was constructed from four fragments (Example 2), appropriate primers were used to yield four PCR fragments encompassing the three regions of the plasmid. All these different constructions are detailed hereinbelow. For the three, four and five fragment construction, the following was carried out:

Example 1

Assembly of three DNA fragments

The plasmid illustrated in Fig. 2 was assembled by the joining of three independently produced DNA fragments. These three DNA fragments are:

a) a DNA fragment of 1739 base pairs (bp) containing the Amp^r gene and the ColE1-ORI region;

b) a DNA fragment of 1466 bp containing the Tet^r gene; and

c) a DNA fragment of 745 bp containing the Cm^r gene.

These fragments are illustrated schematically in Fig. 2 showing their relative positioning one to the other, namely, that the Cm^r fragment was to be connected at its one end to one end of the Tet^r fragment and at its other end to one end of the Amp^r + ColE1-ORI fragment, and likewise, the other ends of the Tet^r and Amp^r + ColE1-ORI fragments were to be connected to each other to provide for a circular DNA molecule, being the desired plasmid, having the above predetermined order of the three fragments.

To produce the above three DNA fragments, the aforementioned PCR procedure was carried out using the following primers and template DNA:

a) The 1739 bp Amp^r + ColE1-ORI DNA fragment was synthesized by the PCR procedure using primers SEQ. ID NO. 3 and SEQ. ID NO. 4 and pBR322 as the template DNA. The concentrations of the primers and template DNA, as well as the other PCR conditions, are as indicated in the

above table under "Materials and Methods". The relative direction of the primers with respect to the synthesis of the Amp^r + ColE1-ORI fragment as it is positioned in the completed plasmid product is as depicted schematically in Fig. 2. Thus, primer SEQ. ID NO. 3 was synthesized to have a predetermined sequence so as to provide for the desired junction region between the Amp^r + ColE1-ORI fragment and the Cm^r fragment, and primer SEQ. ID NO. 4 was synthesized to have a predetermined sequence so as to provide for the desired junction between the Amp^r + ColE1-ORI fragment and the Tet^r fragment.

b) The 1466 bp Tet^r DNA fragment was synthesized by the PCR procedure using primers SEQ. ID NO. 5 and SEQ. ID NO. 6 and pBR322 as the template DNA. The concentrations of the primers and template DNA, as well as the other PCR conditions, are as indicated in the above table under "Materials and Methods". The relative direction of the primers with respect to the synthesis of the Tet^r fragment as it is positioned in the completed plasmid product is as depicted schematically in Fig. 2. Thus, primer SEQ. ID NO. 5 was synthesized to have a predetermined sequence so as to provide for the desired junction region between the Tet^r fragment and the Cm^r fragment, and primer SEQ. ID NO. 6 was synthesized to have a predetermined sequence so as to provide for the desired junction between the Tet^r fragment and the Amp^r + ColE1-ORI fragment.

c) The 745 bp Cm^r DNA fragment was synthesized by the PCR procedure using primers SEQ. ID NO. 7 and SEQ. ID NO. 8 and pACYC184 as the template DNA. The concentrations of the primers and the template DNA, as well as the other PCR conditions, are as indicated in the above table under "Materials and Methods". The relative direction of the primers with respect to the synthesis of the Cm^r fragment as it is positioned in the completed plasmid product is as depicted schematically in Fig. 2. Thus, primer SEQ. ID NO. 7 was synthesized to have a predetermined sequence so as to provide for the desired junction region between the Cm^r fragment and the Tet^r fragment, and primer SEQ. ID

NO. 8 was synthesized to have a predetermined sequence so as to provide for the desired junction between the Cm^r fragment and the Amp^r + ColE1-ORI fragment.

The sequences of all of the above primers are as follows:
All of the primer sequences given herein are written in the 5' to 3' direction. The arrows in the sequences below indicate the junction points.

a) primers SEQ. ID NO. 3 and SEQ. ID NO. 4:

part of Cm^r region sequence — part of Amp^r region sequence

primer SEQ. ID NO. 3: ATTGGTGCCCTTAAACGCCTG↓AACGCAGGAAAGAACATGTG
(also designated 4142)

part of Tet^r region sequence — part of Amp^r region sequence

primer SEQ. ID NO. 4: AGCGTTGGGTCCTGGCCA↓AAGAGTATGAGTATTCAACA
(also designated 3884)

b) primers SEQ. ID NO. 5 and SEQ. ID NO. 6:

part of Cm^r region sequence — part of Tet^r region sequence

primer SEQ. ID NO. 5: AGCTCCTGA↓TTCTCATGTTTGACAGCTTATC
(also designated 31160)

part of Amp^r + ColE1-ORI region sequence — part of Tet^r region sequence

primer SEQ. ID NO. 6: ATACTCTT↓TGGCCAGGACCCAACGCTGCCC
(also designated 30397)

c) primers SEQ. ID NO. 7 and SEQ. ID NO. 8:

part of Tet^r region sequence — part of Cm^r region sequence

primer SEQ. ID NO. 7: AAACATGAGAA↓TCAGGAGCTAAGGAAGCTAAAATG
(also designated 3595)

The Amp region — The Cm region

primer SEQ. ID NO. 8: ATGTTCTTTCCTGCGTT↓CAGGCGTTTAAGGGCACCAATAAC
(also designated 4143)

The above primer sequences were determined from the known sequences of pBR322 and pACYC184 (the full sequence of these plasmids can be

accessed from the GenBank database under accession Nos. J01749 and X06403, respectively). Accordingly, for the preparation of the Tet^r and Amp^r + ColE1-ORI PCR fragments, the pBR322 plasmid was used as template. These primer sequences were chosen to provide for the predetermined desired junction regions between the above three DNA fragments to be PCR synthesized and then joined together. It should be appreciated, however, that different primers may have been synthesized, namely, primers having sequences with more or less of the sequence information from each gene region that it was desired to join together. Hence, the above sequences are but examples of any of a number of suitable sequences, all of which will have sequence information from both gene regions it is desired to join together, with the desired junction point being situated at some point along the sequence. Hence, it should also be understood that the chosen junction region was designed irrespective of restriction endonuclease sites or any other sequence features within this DNA region to illustrate that any DNA sequence encompassing the desired junction region between two gene regions would be acceptable as a junction region sequence.

For the synthesis of the above primers, standard automated procedures to produce polynucleotide oligomers were employed, together with the associated apparatus (Applied Biosystems, U.S.A.), and by also following the manufacturer's instructions.

The above primers were synthesized so as to provide for the PCR products having at their terminal ends the sequence necessary to provide for the desired junction regions.

Thus, for the Cm^r-Tet^r region junction, the following sequence was the chosen one (from a number of possibilities readily apparent to all of skill in the art) to be present at one end (the 5' end) of the Tet^r PCR fragment, and at one end (the 5' end) of the Cm^r PCR fragment. Such a sequence

thus provides for complete homology at these ends of the two PCR fragments to be joined together, and in this case, the connection was chosen to be a 5' end-5' end connection, and this in view of the sense direction of both the Tet^r gene and the Cm^r gene inclusive of their promoters.

5' end of the Cm^r region (Cm^r upstream) — desired junction point — 5' end of the Tet^r region (Tet^r upstream)

5'..CCATTTTAGCTTCCTTAGCTCCTGA↓TTCTCATGTTTGACAGCTTATCATC..3'
3'..GGTAAAATCGAAGGAATCGAGGACT AAGAGTACAAACTGTCGAATAGTAG..5'

The underlined sequences represent the sequences of primers SEQ. ID NO. 5 and SEQ. ID NO. 7, which are used to synthesize this junction region for the Tet^r and Cm^r PCR fragments, respectively. In the upper strand (5'-3' direction), there is underlined the primer SEQ. ID NO. 5 sequence, and in the lower strand (3'-5' direction), there is underlined the primer SEQ. ID NO. 7 sequence. It should be noted, as indicated above, that the Tet^r fragment is produced only from primers SEQ. ID NO. 5 and SEQ. ID NO. 6, and the Cm^r fragment is produced only from primers Nos. SEQ. ID NO. 7 and SEQ. ID NO. 8. However, the junction region between the Tet^r and Cm^r fragments will be the homologous region indicated above, present on both fragments as a result of the respective use of the primers SEQ. ID NO. 5 and SEQ. ID NO. 7. Accordingly, the 5' end of the Tet^r PCR fragment will be (starting from the primer SEQ. ID NO. 5):

5' AGCTCCTGATTCTCATGTTTGACAGCTTATCATC . . . .3'
3' TCGAGGACTAAGAGTACAAACTGTCGAATAGTAG . . . .5'

Likewise, the 5' end of the Cm^r PCR fragment will be (starting from primer SEQ. ID NO. 7):

5' AAACATGAGAATCAGGAGCTAAGGAAGCTAAAATG . .3'
3' TTTGTACTCTTAGTCCTCGATTCCTTCGATTTTAC. . 5'

Hence, both the Tet^r and Cm^r PCR fragments share a common homologous region at their 5' ends, this being indicated by the underlining in both above sequences of the 5' ends of these PCR fragments, this homologous region thus included in the above chosen junction region. As illustrated, this common homologous region at the ends of the Tet^r and Cm^r PCR fragments is of length of 20 nucleotides, this being in the desired range of greater than 15 nucleotides. Upon treatment of these PCR fragments with Exo III, the following single-stranded 5' overhangs are produced:

The Tet^r PCR fragment after Exo III digestion under conditions that will yield degradation of, for example, 22 nucleotides:

5' A G C T C C T G A T T C T C A T G T T T G A C A G C T T A T C A T C . . . 3'
3' (22 nucleotides digested by Exo III) G T C G A A T A G T A G . . . 5'

The Cm^r PCR fragment after exonuclease digestion under similar conditions that will yield degradation of, for example, 23 nucleotides:

5' A A A C A T G A G A A T C A G G A G C T A A G G A A G C T A A A A T G . . . . 3'
3' (23 nucleotides digested by Exo III) C T T C G A T T T T A C . . . . 5'

Thus, the resulting 5' overhangs on both the Tet^r and Cm^r PCR fragments following Exo III digestion are complementary and will allow for a complementary interaction to occur between them via hydrogen bonding and thereby to ensure the joining of these fragments at these 5' ends.

For illustrative purposes, the following is the schematic joining of the above Tet^r and Cm^r fragments via their complementary overhangs:

Cm^r upstream region (5') 3'..5' | junction point between Cm^r and Tet^r | Tet^r upstream (5') region

5'. . . T C A G C T C C T G A ↓ T T C T C A T G T T T G A C A G C T T . . . . . 3'
3'. . . A G G A A T C G A G G A C T A A G A G T A C A A A G T C G A A . . . . . 5'
5' 3'

In the above junction region between the Tet^r and Cm^r fragments, the simply underlined bases are from the above Exo III-digested Tet^r fragment and the dotted underlined bases are from the Exo III-digested Cm^r fragment. As is apparent from the above junction region, this has the sequence of the above-noted chosen junction region, wherein the chosen junction point (see arrow above between the "A" and the "T") is within the complementary 5' overhang region present on both the Tet^r and Cm^r fragments.

In view of the fact that the original primer sequences were derived only from the Tet^r and Cm^r genes, with no introduction of any additional ("linker") DNA sequences whatsoever, the above junction is a "seamless" one between only Tet^r and Cm^r gene sequences.

By the same way as noted above for the Cm^r-Tet^r junction region, the junction regions for the other two junctions to yield the desired product were also devised and produced. Namely, for the Amp^r-Tet^r junction region, the following sequence (from a number of possibilities) was chosen:

	desired junction point	
Amp region (bases 4160-4183 in the pBR322 map)		downstream Tet region (3') (bases 1448-1424 in the pBR322 map)

GAATGTTGAATACTCATACTCTT↓TGGCCAGGACCCAACGCTGCCCGAG
CTTACAACTTATGAGTATGAGAA↓ACCGGTCCTGGGTTGCGACGGGCTC

This Amp^r-Tet^r junction region is derived from the sequences of the appropriately designed primers SEQ. ID NO. 6 and SEQ. ID NO. 4 noted hereinabove. Primer SEQ. ID NO. 6, which is used for amplifying the Tet^r fragment, includes sequences of the 5' Amp^r region found right next to the junction point (the 8 nucleotides left of the junction point on the upper strand shown above). Primer SEQ. ID NO. 4, which is used for amplifying the Amp^r fragment, includes sequences of the Tet^r region, found right next to the junction point (the 18 nucleotides right of the junction point on the lower strand shown above).

Hence, once the Tet^r and Amp^r fragments are digested by the Exo III, the Tet^r fragment will have a 5' overhang complementary to the 5' overhang on the Amp^r fragment, that upon joining, by the above joining reaction, will yield the desired junction region noted above. Here, too, this junction region is a "seamless" connection between only Tet^r gene sequences and Amp^r + ColE1-ORI region sequences having no foreign ("linker") DNA whatsoever.

Similarly, by the same way noted above for the Cm^r-Tet^r and Amp^r-Tet^r junction regions, to provide the other (third) junction in this three-fragment circular construct, namely the Amp^r-Cm^r junction region, the same procedure was carried out. The following sequence was chosen (from a number of possibilities) as the Amp^r-Cm^r junction region to join between the 3' end of the Cm^r region and the 3' end of the Amp^r region:

desired junction point

Amp region (bases 2461-2484 in the pBR322 map) — Cm region (3') (bases 3769-3792 in the pACYC184 map)

TGCTCACATGTTCTTTCCTGCGTT↓CAGGCGTTTAAGGGCACCAATAAC
ACGAGTGTACAAGAAAGGACGCAA GTCCGCAAATTCCCGTGGTTATTG

This Amp^r-Cm^r junction region is derived from the sequences of the appropriately designed primers SEQ. ID NO. 8 and SEQ. ID NO. 3 noted hereinabove. Primer SEQ. ID NO. 3, which is used for amplifying the Amp^r fragment, includes sequences from the 3' Cm^r region found right next to the junction point (the 21 nucleotides right of the junction point on the lower strand shown above). Primer SEQ. ID NO. 8, which is used for amplifying the Cm^r fragment, includes sequences from the 3' Amp^r region, found right next to the junction point (the 17 nucleotides left of the junction point on the upper strand shown above)..

Hence, once the Amp^r and Cm^r fragments are digested by the Exo III, the Amp^r fragment will have a 5' overhang complementary to the 5' overhang

on the Cm^r fragment, that upon joining, by the above joining reaction, will yield the desired junction region noted above. Here too, this junction region is a "seamless" connection between only Amp^r + ColE1-ORI region sequences and Cm^r gene sequences having no foreign ("linker") DNA whatsoever.

Furthermore, it should be noted, as is readily apparent from the sequences of the above-listed primers, that the primers used for the synthesis of each fragment are each unique in their sequence. Each of the above three PCR fragments are synthesized from two different primers, the sequence of which yields essentially no homology between the two ends of the fragment, and thus, upon Exo III degradation, the resulting 5' overhangs on each end of a given PCR fragment will not be complementary to each other, preventing this fragment from joining to itself or to another identical fragment. By designing the primer sequences so that for each intended junction there is a different pair of primers, one primer to define the sequence of one PCR fragment at one of its ends and the other primer to define the sequence of another PCR fragment at one of its ends, and wherein these two ends of these two PCR fragments are homologous due to these primer sequences, there is provided the way for specifically joining these two ends together as noted above.

Likewise, each such pair of primers for each intended junction region was unique, thereby ensuring that only those desired predetermined ends of two PCR fragments were capable of joining to each other. This design, therefore, provides for the specific, directional and seamless joining of the various fragments to each other to form a DNA construct having the desired order of its constitute parts.

Following the preparation of the desired construct described above from the three PCR fragments, this construct was analyzed for its biological activity, namely, whether or not it could confer resistance to all three

antibiotics when introduced into bacterial cells. This transformation of the bacterial cells, for example, the electropotent DH10B *E. coli* cells obtained from a commercial supplier, is preferably performed by electroporation. Many other suitable methods and cells may be employed, as is readily apparent to all of skill in the art, in view of the substantial publication of such methods and cells (see, for example, the above referenced Sambrook et al., 1989).

In the present analysis, the above preferred technique of electroporation of electropotent DH10B *E. coli* cells was performed using a 2 µl aliquot of a 5 µl final product containing the DNA construct (see procedure under "Materials and Methods" above). After electroporation, the cells were first plated on agar plates containing ampicillin. The results revealed more than 1000 colonies on these plates, indicating that more than 1000 originally transformed cells received a DNA construct having at least an active Amp^r gene. Of these Amp^r colonies, 40 were chosen at random, as a test sample, and were plated on both tetracycline- and chloramphenical-containing agar plates. All 40 of these test colonies grew on these plates as well, indicating that they were also Tet^r and Cm^r. Hence, it is concluded that at least these 40 colonies received an intact construct in which all of the Tet^r, Cm^r and Amp^r genes were intact and fully expressable. Some of the colonies were tested further by PCR, as indicated above. The PCR bands that appeared were of the expected sizes.

Example 2:

Construction of a plasmid from 4 individual DNA fragments

The same plasmid depicted in Fig. 2 and described in Example 1 above was constructed from four independent DNA fragments, each produced by the PCR procedure. All of the procedures for the PCR synthesis of the individual fragments and the subsequent Exo III digestion thereof and the joining thereof to form a DNA construct are essentially as set forth in Example 1 above. The only difference is that the Tet^r fragment was

designed to be constructed from two separate subfragments, the Tet A and Tet B subfragments, which, when joined together, will constitute the complete Tet^r fragment. Hence, in this 4-fragment construct, the Cm^r fragment and the Amp^r + ColE1-ORI fragment are produced exactly as in Example 1 above from exactly the same primers. The Tet A and Tet B subfragments are indicated schematically in Fig. 2. The Tet A fragment was chosen to be an 857 bp fragment carrying the 5' region of the Tet^r gene and the Tet B subfragment was chosen to be a 632 bp fragment carrying the 3' region of the Tet^r gene. Accordingly, it was necessary to synthesize two additional primers, Nos. SEQ. ID NO. 9 and SEQ. ID NO. 10, which will constitute the joining region of the two Tet subfragments, this being the internal Tet^r gene junction region. Thus, the Tet^r A subfragment is synthesized from primers SEQ. ID NO. 5 (noted above) and SEQ. ID NO. 10, the sequence of which is shown below. The Tet B subfragment is synthesized from primers SEQ. ID NO. 6 (noted above) and SEQ. ID NO. 9 (set forth below).

The sequences of these primers SEQ. ID NO. 9 and SEQ. ID NO. 10 are as follows:

Tet^r internal region - sense strand

Primer SEQ. ID NO. 9: A T C G G C C T G T C G C T T G C G G T A T T C G
(also designated 25586)

Tet^r internal region - antisense strand

Primer SEQ. ID NO. 10: A T A C C G C A A G C G A C A G G C C G A T C A T C G
(also designated 27336)

These additional primers SEQ. ID NO. 9 and SEQ. ID NO. 10 are also shown in Fig. 2, together with arrows indicating their relative direction with respect to the final plasmid product.

As explained in Example 1 above, PCR synthesis of the Tet^r A and Tet^r B fragments will provide for the Tet^r A fragment having a sequence at its 3' end being the sequence of primer SEQ. ID NO. 10, which includes

sequence information of the junction region. Likewise, the Tet^r B fragment will have at its 5' end the sequence of primer SEQ. ID NO. 9, which has the information of the junction region. As a result, the Tet^r A 3' end will be homologous to the Tet^r B 5' end, thereby providing for the joining only at these ends of the Tet^r A to the Tet^r B fragments (this following the Exo III digestion and subsequent joining reaction). Once joined, the following predetermined junction region, being the internal Tet^r junction region, will have the following sequence:

internal Tet region - fusion zone

```
820 5'  CGATGATCGGCCTGTCGCTTGCGGTATTCG  3'  850
    3'  GCTACTAGCCGGACAGCGAACGCCATAAGC  5'
```

It should be noted that the above "820" and "850" represent the map positions of the above junction region with respect to the pBR322 map, the pBR322 being of course the template for the production of the above Tet^r A and Tet^r B fragments.

Furthermore, it should be noted that the above additional primers SEQ. ID NO. 9 and SEQ. ID NO. 10 are unique with regards to their sequence, with the result that none of the other PCR fragments in this 4-fragment construct will be able to interact in a non-specific fashion with the Tet^r A and the Tet^r B fragments, and only the Tet^r A and the Tet^r B fragments will be able to join to each other in the specific manner indicated above, namely, the 3' end of the Tet^r A fragment will join only to the 5' end of the Tet^r B fragment.

Thus, following the PCR construction of the Tet^r A and the Tet^r B fragments, these fragments were added in a mixture to the above-noted (Example 1) Cm^r fragment and Amp^r + ColE1-ORI fragment providing a mixture of four individual PCR fragments. This mixture was then subjected to Exo III digestion, heating, slow cooling, T7 DNA polymerase

and T4 DNA ligase treatment to yield a final product which is essentially the construct shown in Fig. 2.

Bacterial cells were transformed exactly according to the procedures in Example 1. The transformed cells were plated on agar plates containing ampicillin. The results revealed that 320 independent colonies were obtained on the ampicillin-containing plates, indicating that at least as far as the Amp^r gene is concerned, the cells from which these colonies arose all received an intact and active Amp^r gene. Of these 320 Amp^r colonies, 50 were chosen at random and tested both on tetracycline and chloranphenicol-containing plates. The results revealed that 47 of these were also both tetracycline- and chloranphenicol-resistant, while 3 were only tetracycline-resistant, but sensitive to chloranphenicol. Hence, it could be concluded that a very high percentage of the tested colonies showed that the originally transformed cells received an intact and fully active plasmid construct of the type depicted in Fig. 2, namely, that they all had intact and active Amp^r, Tet^r and Cm^r genes. No PCR testing was performed on these colonies.

The above results, therefore, clearly indicate that the above procedure to prepare a plasmid construct from four independent PCR-produced fragments, is highly specific and highly efficient.

Example 3

Construction of a plasmid from 5 independent DNA fragments

Using the same procedures set forth in Examples 1 and 2 above, the DNA construct illustrated in Fig. 2 was constructed from five independent PCR-produced DNA fragments. The only difference in this new 5-fragment construct and the one noted in Example 2 above, was that the Cm^r region was devised to be constructed from two separate fragments, to be joined together at an internal junction in the final construct. Hence,

the Amp^r + ColE1-ORI fragment was produced exactly as set forth in Example 1, the Tet^r A and Tet^r B fragments were produced exactly as set forth in Example 2 above, to provide three of the five fragments for this construct, while the Cm^r fragment was now constructed in the form of two subfragments, the Cm^r A and Cm^r B subfragments. The Cm^r A subfragment was chosen to be a 475 bp fragment, and the Cm^r B was chosen to be a 271 bp fragment. For the construction of these subfragments, two additional primers, SEQ. ID NO. 1 and SEQ. ID NO. 2 were synthesized, the sequences of which will provide for the junction region between the Cm^r A and the Cm^r B fragments.

Thus, the Cm^r A subfragment was synthesized using primers SEQ. ID NO. 7 (noted above in Example 1) and the additional new primer SEQ. ID NO. 1 (sequence shown below), and the Cm^r B fragment was synthesized from primer SEQ. ID NO. 8 (as set forth in Example 1 above) and the additional new primer SEQ. ID NO. 2 (sequence set forth below). The sequences of these primers were of course determined from the known sequence of the plasmid pACYC184, which was used as the template to produce these PCR fragments. The sequences of these primers SEQ. ID NO. 1 and SEQ. ID NO. 2 are as follows:

Primer SEQ. ID NO. 1:
(also designated 27337)

Cm^r internal region - noncoding strand

ATTGGCTG AGACGAAAAA CATATTCTC 27 bp

Primer SEQ. ID NO. 2
(also designated 25587)

Cm^r internal region - coding strand

ATATGT TT T TCGTCTCAGCCAATCC 25 bp

Thus, using the above additional primers, the two resulting Cm^r A and Cm^r B fragments will have homologous ends, corresponding to the desired, predetermined junction region to be formed between these two fragments. As mentioned previously, with respect to all of the other primers, these

additional primers are also unique in their sequence and will provide only for homology at these two ends of the Cm^r A and Cm^r B fragments, ensuring that these fragments will only join to each other at these ends. More specifically, the 3' end of the Cm^r A fragment will have a sequence of primer SEQ. ID NO. 1, which contains sequence information of the junction region and the 5' end of the Cm^r B fragment will have the sequence of primer SEQ. ID NO. 2, which contains the sequence information of the junction region as well. The other ends of the Cm^r A and Cm^r B fragments, namely, the 5' end of the Cm^r A fragment, will be homologous to the 5' end of the Tet^r A fragment, and the 3' end of the Cm^r B fragment will be homologous to the 5' end of the Amp^r fragment.

Based on the above, when the Cm^r A and the Cm^r B fragments join together, the following predetermined chosen joining region will be obtained:

```
5' GGATTGGCTGAGACGAAAAACATATTCTT 3'
3' CCTAACCGACTCTGCTTTTTGTATAAGAG 5'
```

Accordingly, following the PCR preparation of the five separate fragments, namely, the two Tet fragments, Tet^r A and Tet^r B, the two Cm^r fragments, the Cm^r A and Cm^r B fragments, as well as the single Amp^r + ColE1-ORI fragment, all of these fragments will then be mixed together, treated with Exo III, heated, cooled, and subsequently treated with the T7 DNA polymerase and T4 DNA ligase, following the same procedures as set forth in Examples 1 and 2 above, to yield a final product, which is the construct illustrated in Fig. 2.

Upon analysis of the biological activity of the above 5-fragment construct by using it to transform bacteria by the same procedures detailed in Examples 1 and 2 (and under "Materials and Methods"), it was found that eight independent colonies grew on Agar plates containing ampicillin indicating that all of these had received at least an intact and active Amp^r

gene. Of these eight Amp^r colonies, six were also shown to be both Tet^r and Cm^r by virtue of their ability to grow on both tetracycline and chloramphenicol-containing Agar plates. These results thus indicate that even for the 5-fragment construct the procedure of the present invention has relatively high efficiency and that most of the positively transformed colonies received an intact and active plasmid construct containing all three antibiotic resistance genes. The six colonies that were resistant to all three antibiotics were tested further by the PCR tests mentioned above. The results (not shown) confirmed that they contained the required constructs.

It should be noted that for the constructs of Examples 2 and 3, wherein the Tet^r PCR fragment was prepared as two subfragments Tet^r A and Tet^r B (Examples 2 and 3) and wherein the Cm^r PCR fragment was prepared as two subfragments Cm^r A and Cm^r B (Example 3), the chosen junction region between the two subfragments, namely, the internal Tet^r and Cm^r junction regions, was a choice from many possibilities, as essentially this junction region could be anywhere along the Tet^r or Cm^r region. Hence, for such internal junctions, the flexibility of choice is even greater than for the junctions between different gene regions, e.g., the junctions between the Cm^r and Tet^r regions.

Example 4

Comparing the efficiency of constructing plasmids with fragments containing overlaps of 12 bp compared to fragments containing overlaps of around 20 bp.

The plasmids to be constructed were designed to have three or more different regions, each to be prepared separately by PCR amplification and then joined in a specific directional fashion to provide a circularized plasmid as the end-product. This required the initial preparation of specific primers, the sequence of which was to provide the basis on which the

various PCR fragments, constituting the various regions of the plasmid, would be able to combine with each other to ultimately connect all the regions in the specific directional manner. Each primer consisted of 2 regions: A 3' region complementary to the fragment to be amplified and a 5' region complementary to the fragment to be connected to. In order to show the advantage of the present invention, two sets of plasmids were constructed. In the first set the homology between the fragments was of 12 bp (hereinafter 12 bp overlap). In the second set the homology between the fragments was of 19-36 bp (hereinafter 20 bp overlap). Note that in this example the overhangs were generated utilizing the exonucleolytic activity of T4 DNA polymerase (instead of using Exonuclease III as in the previous examples). Also note, that in this example there is no use of ligase prior to the transformation, and the covalent linking between the fragments is carried out in-vivo, by the endogenous ligating enzymes. Plasmids were constructed from 3, 4 and 5 fragments, as shown below.

Plasmids containing 3 fragments (hereinafter pCATK3, Fig. 3A) were constructed from the following fragments: An approximate 1700 bp fragment which contained the ampicillin resistance gene (Amp^r) and the ColEI origin of replication, an approximate 1400 bp fragment which contained the tetracycline resistance gene (Tet^r), and an approximate 1900 bp fragment which contained both the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Kn^r).

Plasmids containing 4 fragments (hereinafter pCATK4, Fig. 3B) were constructed from the following fragments: An approximate 1700 bp fragment which contained the Amp^r and the ColEl origin of replication, an approximate 1400 bp fragment which contained the Tet^r, an approximate 700 bp fragment which contained the Cm^r and an approximate 1200 bp fragment which contained the Kn^r.

Plasmids containing 5 fragments (hereinafter pCATK5, Fig. 3C) were constructed from the following fragments: an approximate 1700 bp fragment which contained the Amp^r and the ColE 1 origin of replication, an approximate 800 bp fragment which contained the 5' terminal part of the Tetracycline resistance gene ($TetA^r$), an approximate 600 bp fragment which contained the 3' terminal part of the Tetracycline resistance gene ($TetB^r$), an approximate 700 bp fragment which contained Cm^r and an approximate 1200 bp fragment which contained the Kn^r. The fragments length is approximate.

All the plasmids to be constructed in this example include the genes conferring resistance to the following four antibiotics resistance: Amp^r, Tet^r, Cm^r, and Kn^r. Accordingly, it is possible to select for colonies carrying plasmid by plating cells on LB agar plates containing ampicillin and the screening for those host cells transformed by the correct plasmids is carried out by growing the cells in the presence of all four antibiotics.

Materials and Methods for Example 4

Reagents were obtained from the following sources - Taq DNA polymerase from Boehringer Mannheim, dNTP'S and T4 polymerase from Promega and reagents grade chemicals were from Sigma, Merck and Frutarom.

The appropriate fragments were prepared using a standard PCR procedure. The PCR was performed in a RoboCycler gradient 96 (Stratagene) for 30 cycles, each cycle consisting of a denaturing step at 94°C for 45 seconds, a primer annealing step at 55°C for 60 seconds and an extension step at 72°C for 120 seconds. The first denaturing step at 94°C was for 120 seconds and the last extension step at 72°C was for 300 seconds. The exact amount of the reaction ingredients is given below.

	amount in µl
primer[1]	20
primer[1]	20
10xbuffer	40
dNTP's[3]	32
DNA[2]	0.5
H_2O.	286
Taq polymerase[4]	1.5
Final volume	400

1. Primers were diluted according to their size in accordance to: 0. 1 pmole/µl for 20 bp long primer.
2. The templates were: pBR 322, PACYC184, pACYC177 (all available from New England Biolabs) and PCATK (which was build at Gesher Israel Advanced Biotechs). The concentration of the templates was 0.1 µg/µl.
3. The dNTP's stock solution concentration was 2.5 mM each.
4. The Taq polymerase stock solution was 5 U/µl.

After the PCR, the fragments were run on 1 % agarose gel, cut out from the gel and cleaned using 'Prep-A-Gene DNA Purification System' (Bio-Rad).

For the construction itself, 0.15 pmole of each fragment were mixed together with T4 DNA polymerase in the absence of dNTP's at 37°C for 2 minutes in 33mM Tris-acetate (pH 7-9) containing 66 mM KOAc, 10 mM $Mg(OAc)_2$, 0.5mM DTT and 100µg/ml BSA. Following enzyme. inactivation at 70°C for 10 minutes, the mixture was incubated at 37°C for 2 hours. After the annealing step, dNTP's (final concentration 0. l mM), DTT (final concentration 0.5mM) and 0.1 u/µl T4 polymerase were added and the mixture was incubated at 37°C for 30 minutes and 1 µl of the mixture was transformed into ElectroMAX DH1OB cells (GIBCO BRL) by electroporation using Bio-Rad *E. coli* pulser (Bio-Rad), and plated on agar plates containing ampicillin.

The day after the transformation, colonies were counted. A sample of the colonies was checked for the presence of the relevant antibiotic resistance by plating on agar LB plates containing all four antibiotics.

Sequence of primers used in the example

Primers for the amplification of fragments containing 12 bp overlap:

Primers for the Amp^r fragment:

Primer SEQ. ID NO. 11
(also designated 26602)
Cm region Amp *region*
CGCCTG↓AACGCAGGAAAGAACATGTG

Primer SEQ. ID NO. 12
(also designated 31230)
Tet region *Amp region*
GGCCA↓AAGAGTATGAGTATTCAACATTTCCG

Primers for the Tet^r fragment:

Primer SEQ. ID NO. 13
(also designated 29263)
Amp region *Tet region*
TACTCTT↓TGGCCAGGACCCAACGCTGCCC

Primer SEQ. ID NO. 14
(also designated 31201)
Kn region *Tet region*
GCTTTGTTG↓TTCTCATGTTTGACAGCTTATC

Primers for the Cm^r fragment:

Primer SEQ. ID NO. 15
(also designated 28405)

Kn region Cm region

AGTC↓TCAGGAGCTAAGGAAGCTAAAATG

Primer SEQ. ID NO. 16
(also designated 30606)

Amp region *Cm region*

TGCGTT↓CAGGCGTTTAAGGGCACCAATAAC

Primers for the Kn^r fragment:

Primer SEQ. ID NO. 17
(also designated 25891)

Tet region *Kn region*

GAA↓CAACAAAGCCACGTTGTGTCTC

Primer SEQ. ID NO. 18
(also designated 30509:

Cm region *Kn region*

GCTCCTGA↓GACTCATACCAGGCCTGAATCG

Primers for the Cm^r-Kn^r fragment:

Primer SEQ. ID NO. 17 and Primer SEQ. ID NO. 16.

Primers for the amplification of fragments containing around 20 bp overlap:

Primers for the Amp^r fragment:

Primer SEQ. ID NO. 19
(also designated 4086)

Cm region *Amp region*

TTGGTGCCCTTAAACGCCTG↓AACGCAGGAAAGAACATGTG

Primer SEQ. ID NO. 20
(also designated 37112)

Tet region *Amp region*

GCGTTGGGTCCTGGCCA↓AAGAGTATGAGTATTCAACA

Primers for the Tet^r fragment:

Primer SEQ. ID NO. 13 and Primer SEQ. ID NO. 14

Primers for the TetA^r fragment:

Primer SEQ. ID NO. 21
(also designated 27438)

Tet region

ATACCGCAAGCGACAGGCCGATCATCG

and Primer SEQ. ID NO. 14

Primers for the TetB^r fragment:

Primer SEQ. ID NO. 22
(also designated 25771)

Tet region

ATCGGCCTGTCGCTTGCGGTATTCG

and Primer SEQ. ID NO. 13

Primers for the Cm^r fragment:

Primer SEQ. ID NO. 23
(also designated 36143)

Kn region *Cm region*

CTGGTATGAGTC↓TCAGGAGCTAAGGAAGCTAAAATG

Primer SEQ. ID NO. 24
(also designated 4085)

Amp region Cm region

TGTTCTTTCCTGCGTT↓CAGGCGTTTAAGGGCACCAATAAC

Primers for the Kn^r fragment:

Primer SEQ. ID NO. 25
(also designated 32199)

Tet region Kn region

AACATGAGAA↓CAACAAAGCCACGTTGTGTCTC

and Primer SEQ. ID NO. 18

Primers for the Cm^r-Kn^r fragment:

Primer SEQ. ID NO. 25 and Primer SEQ. ID NO. 24

Results

After the amplification of the fragments they were mixed and their 5' overhangs exposed utilizing T4 DNA polymerase. After an annealing time period, dNTP's (final concentration 0. 1 mM), DTT (final concentration 0.5mM) and 0.1 u/µl T4 polymerase were added and the mixture was incubated at 37°C for 30 minutes, and transformed into bacterial cells (as described hereinabove).

The following tables summarize the results of the experiments. After the transformation the cells were first plated on LB agar plates containing ampicillin and the number of colonies counted (see "No. of colonies" in the tables). Of these, a sample (or all the colonies available) were further analyzed by plating on LB agar plates containing all four appropriate antibiotics - ampicillin, tetracycline, chloramphenicol, and kanamycin. The results of these platings are summarized in the tables (see "estimated % of correct colonies").

Plasmids constructed from 3 fragments

		12 bp overlap		20 bp overlap	
T4 pol. conc. (u/µl)	Experi-ment no.	No. of colonies	estimated % of correct colonies[1]	No of colonies	estimated % of correct colonies[1]
0.09	1	1	0	196	70
	2	0	0	328	85
	3	2	0	176	90
	4	1	0	494	70
0.19	1	1	0	245	90
	2	1	0	104	100
	3	1	0	99	75

1. 20 colonies (or the total number of the colonies, if there were less than 20 colonies) were randomly selected and checked for the presence of the relevant antibiotic resistance by plating on agar LB plates containing ampicillin, tetracycline, chloramphenicol and kanamycin.

Plasmids constructed from 4 fragments

		12 bp overlap		20 bp overlap	
T4 pol. conc. (u/µl)	Experi-ment No.	No. of colonies	estimated % of correct colonies[1]	No of colonies	estimated % of correct colonies[1]
0.09	1	3	0	70	85
	2	0	0	25	95
0.19	1	2	0	36	55

1. 20 colonies (or the total number of the colonies, if there were less than 20 colonies) were randomly selected and checked for the presence of the

relevant antibiotic resistance by plating on agar LB plates containing ampicillin, tetracycline, chloramphenicol and kanamycin.

No constructs assembled from 3 or 4 fragments were obtained when complementary overhangs of 12 bases were used. Therefore, building constructs assembled from 5 fragments having overhangs of 12 bases was not attempted. On the other hand, constructs assembled from five fragments having complementary overhangs of 20 or more bases were produced. The results are shown in the table below.

Plasmids constructed from 5 fragments

		20 bp overlap	
T4 pol. conc. (u/µl)	Experi-ment No.	No of colonies	estimated % of correct colonies[1]
0.09	1	18	22
	2	11	63
0.19	1	4	0

1. The colonies were furtherchecked for the presence of the relevant antibiotic resistance by plating on agar LB plates containing ampicillin, tetracycline, chloramphenicol and kanamycin.

Summary of the experiments

	12 bp overlap		20 bp overlap	
fragment No.[1]	total No. of colonies	estimated % of correct colonies[2]	total No. of colonies	estimated% correct colonies[2]
3	7[3]	0	1642[3]	80
4	5[4]	0	131[4]	79
5	-	-	33[5]	33

1. Fragment No., The number of fragments the plasmid was constructed of.
2. 20 colonies (or the total number of the colonies, if there were less than 20 colonies) were randomly selected and checked for the presence of the

relevant antibiotic resistance by plating on agar LB plates containing ampicillin, tetracycline, chloramphenicol and kanamycin.

3. The number is the sum of 7 independent experiments.
4. The number is the sum of 3 independent experiments.
5. The number is the sum of 3 independent experiments.

Thus, the above experiments of example 4 demonstrate that by using long overhangs in order to join DNA fragments according to the invention, there is provided a highly specific and highly efficient method of construct assmebly.

As shown from the results, plasmids constructed out of 3, 4, and 5 fragments were readily obtained using fragments with overlaps longer than 19 bases. In comparison, when using fragments with overlaps of 12 bases, no correct colonies could be obtained even when constructing a plasmid out of 3 fragments.

The results of this example also illustrate that when using the method of the invention there is no need to ligate the fragments before transforming the constructs into the cells, even when constructing a plasmid out of 5 independent fragments. The covalent connections between the fragments are carried out in vivo, by the ligation enzymes of the cells.

Further, as detailed above in Examples 1-4, the method of the present invention provides for "seamless" joining of the fragments to be joined, as well as a highly specific joining of the fragments in a directional manner, and this due to the choice of the specific primer sequences which are derived from the predetermined or known sequences of the gene regions to be connected together..

All the above description and examples have been given for the purpose of illustration, and are not intended to limit the invention in any way. Many different techniques, starting materials and methods can be employed in

carrying out the claimed method, without departing from the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: Single Step DNA Fragments Assembly

(iii) NUMBER OF SEQUENCES: 25

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATTGGCTGAG ACGAAAAACA TATTCTC 27

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATATGTTTTT CGTCTCAGCC AATCC 25

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATTGGTGCCC TTAAACGCCT GAACGCAGGA AAGAACATGT G 41

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCGTTGGGT CCTGGCCAAA GAGTATGAGT ATTCAACA 38

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGCTCCTGAT TCTCATGTTT GACAGCTTAT C 31

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATACTCTTTG GCCAGGACCC AACGCTGCCC 30

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAACATGAGA ATCAGGAGCT AAGGAAGCTA AAATG 35

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ATGTTCTTTC CTGCGTTCAG GCGTTTAAGG GCACCAATAA C 41

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATCGGCCTGT CGCTTGCGGT ATTCG 25

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATACCGCAAG CGACAGGCCG ATCATCG 27

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 11

CGCCTGAACG CAGGAAAGAA CATGTG 26

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 12

GGCCAAAGAG TATGAGTATT CAACATTTCC G 31

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 13

TACTCTTTGG CCAGGACCCA ACGCTGCCC 29

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 14

GCTTTGTTGT TCTCATGTTT GACAGCTTAT C 31

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 15

AGTCTCAGGA GCTAAGGAAG CTAAAATG 28

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 16

TGCGTTCAGG CGTTTAAGGG CACCAATAAC 30

(2) INFORMATION FOR SEQ ID NO: 17

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 17

GAACAACAAA GCCACGTTGT GTCTC 25

(2) INFORMATION FOR SEQ ID NO: 18

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 18

GCTCCTGAGA CTCATACCAG GCCTGAATCG 30

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 19

TTGGTGCCCT TAAACGCCTG AACGCAGGAA AGAACATGTG 40

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 20

GCGTTGGGTC CTGGCCAAAG AGTATGAGTA TTCAACA 37

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 21

ATACCGCAAG CGACAGGCCG ATCATCG 27

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 22

ATCGGCCTGT CGCTTGCGGT ATTCG 25

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 23

CTGGTATGAG TCTCAGGAGC TAAGGAAGCT AAAATG 36

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 24

TGTTCTTTCC TGCGTTCAGG CGTTTAAGGG CACCAATAAC 40

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO. 25

AACATGAGAA CAACAAAGCC ACGTTGTGTC TC 32

Claims

1. A method for the simultaneous multi-DNA fragment assembly of two or more double-stranded DNA fragments, particularly fragments produced by primer extension reaction and in particular the polymerase chain reaction (PCR) or by restriction cleavage, or the like method, comprising the steps of:

(a) providing for each DNA fragment to be joined to a second DNA fragment and optionally to a third DNA fragment two terminal portions, the first of said two terminal portions, located at one end, being complementary to one of the termini of the second fragment, and the second of said two terminal portions, at the other end, being complementary to one of the termini of the third fragment, the complementary regions being at least 15 nucleotides in length and being designed to be unique so that in a given reaction one such region, at the terminus of a given fragment, is complementary only to the specific terminus on another fragment which it is supposed to join;

(b) carrying out in any suitable order the steps of:

(2) mixing all of the DNA fragments of in a single reaction vessel, and

(2) I. adding thereto the enzyme Exonuclease III, or a functional equivalent thereof (exonuclease), under conditions and for a period of time suitable to provide for the exonuclease digestion which will expose complementary overhangs of at least 15 nucleotides at the ends of said DNA fragments;

II. stopping the exonuclease reaction of (b) by denaturing or inactivating said exonuclease, once enough nucleotides have been removed from the ends of the strands of said DNA fragments to expose said complementary overhangs,

(c) subjecting the exonuclease-digested fragments to conditions suitable to provide for the specific joining between each DNA fragment via the complementary overhangs. and

(d) filling in gaps by adding a DNA polymerase activity and deoxytriphosphate nucleotides.

2. A method according to claim 1, wherein the DNA mixing step is carried out before the exonuclease step.

3. A method according to claim 1, wherein the exonuclease step is carried out before the DNA mixing step.

4. A method according to claims 1-3, wherein the DNA fragments to be joined together are obtained by restriction cleavage of DNA molecules containing said fragments and wherein the restriction enzymes used to provide said DNA fragments are chosen so that each fragment will have the desired first and second terminal portions which are complementary to only one terminal portion of one other fragment.

5. A method according to claims 1-3, wherein the DNA fragments to be joined together are produced by the polymerase chain reaction (PCR), said PCR production of the fragments being by: (a) providing for each DNA fragment to be joined to a second DNA fragment and to a third DNA fragment a pair of primers, one primer of each pair having a portion which is complementary to one terminal portion of said DNA fragment, the second primer of each pair having a portion which is complementary to the other terminal portion of said DNA fragment, and wherein the second portion of each of said primers is complementary to the terminal portion of a different fragment to be joined to the said first fragment in a specific positioned relationship; and (b) amplifying each said DNA fragment by the PCR procedure using said primers, to provide PCR produced DNA fragments, each having the desired first and second terminal portions which are complementary only to one terminal portion of one other fragment.

6. A method according to any one of claims 1-5, wherein the DNA fragments to be joined are a mixture of DNA fragments, some being provided by restriction cleavage and some being provided by a primer extension reaction, all of said fragments having the desired first and second terminal portions which are complementary to only one terminal portion of one other fragment.

7. A method according to claim 6, wherein the primer extension reaction is PCR.

8. A method according to any one of claims 1-7, wherein the covalent connections of the exonuclease-digested DNA fragments are facilitated *in vivo* by transforming and/or transfecting suitable host cells with the DNA constructs following the fill-in step, and whereby the ligation of the fragments occurs endogenously in the host cells by endogenous enzymes.

9. A method according to any one of claims 1-7, wherein the joining of the exonuclease-digested DNA fragments *in vitro* comprises:

(a) heating the reaction mixture after stopping the exonuclease reaction to about 75°C and incubating said mixture for a period of time sufficient to disconnect illegitimate connections between the fragments at said temperature;

(b) slowly cooling the heated mixture of (a) to promote the specific joining of complementary overhangs; and

(c) adding to the cooled mixture of (b) at about 37°C the enzyme T7 DNA polymerase, or T4 DNA polymerase, or a functional equivalent thereof, as well as dNTPs, and optionally also adding the enzyme T4 DNA ligase, under suitable conditions to facilitate filling in and ligation of the strands of the joined fragments.

10. A method according to any one of claims 1-8, wherein the joining of the exonuclease-digested DNA fragments *in vitro* comprises:

incubation of the fragments at 37°C or at a temperature suitable to allow correct hybridization of the overhangs.

11. A method according to any one of claims 1-10, wherein the DNA fragments to be joined are selected from one or more DNA fragments having regulatory function, of the group consisting of promoters, enhancers, terminators, ribosome binding sites, and from one or more DNA fragments encoding proteins of the group consisting of enzymes, cytokines, hormones.

12. A method according to claim 11, wherein the enzymes are selected from the group consisting essentially of citrate synthases, polyketide synthases, and succinyl-CoA-synthetase.

13. A method according to any one of claims 1-12 wherein one or more of said DNA fragments to be joined is a mutant fragment having been subjected to site-directed mutagenesis or mutagenic PCR during its preparation.

14. A method according to any one of claims 1-13 wherein the exonuclease is Exonuclease III.

15. A method according to any one of claims 1-13 wherein the exonuclease is T4 DNA polymerase.

16. An assembled DNA construct whenever prepared by a method according to any one of claims 1-15.

17. An assembled DNA construct according to claim 16, wherein said construct is in the form of a linear DNA molecule.

18. An assembled DNA construct according to claim 16, wherein said construct is in the form of a closed circular DNA molecule.

19. A DNA fragment comprising an overhang of at least 15 nucleotides or an end portion suitable to be converted into such an overhang.

20. A DNA fragment as claimed in claim 19, for use in the method of claim 1.

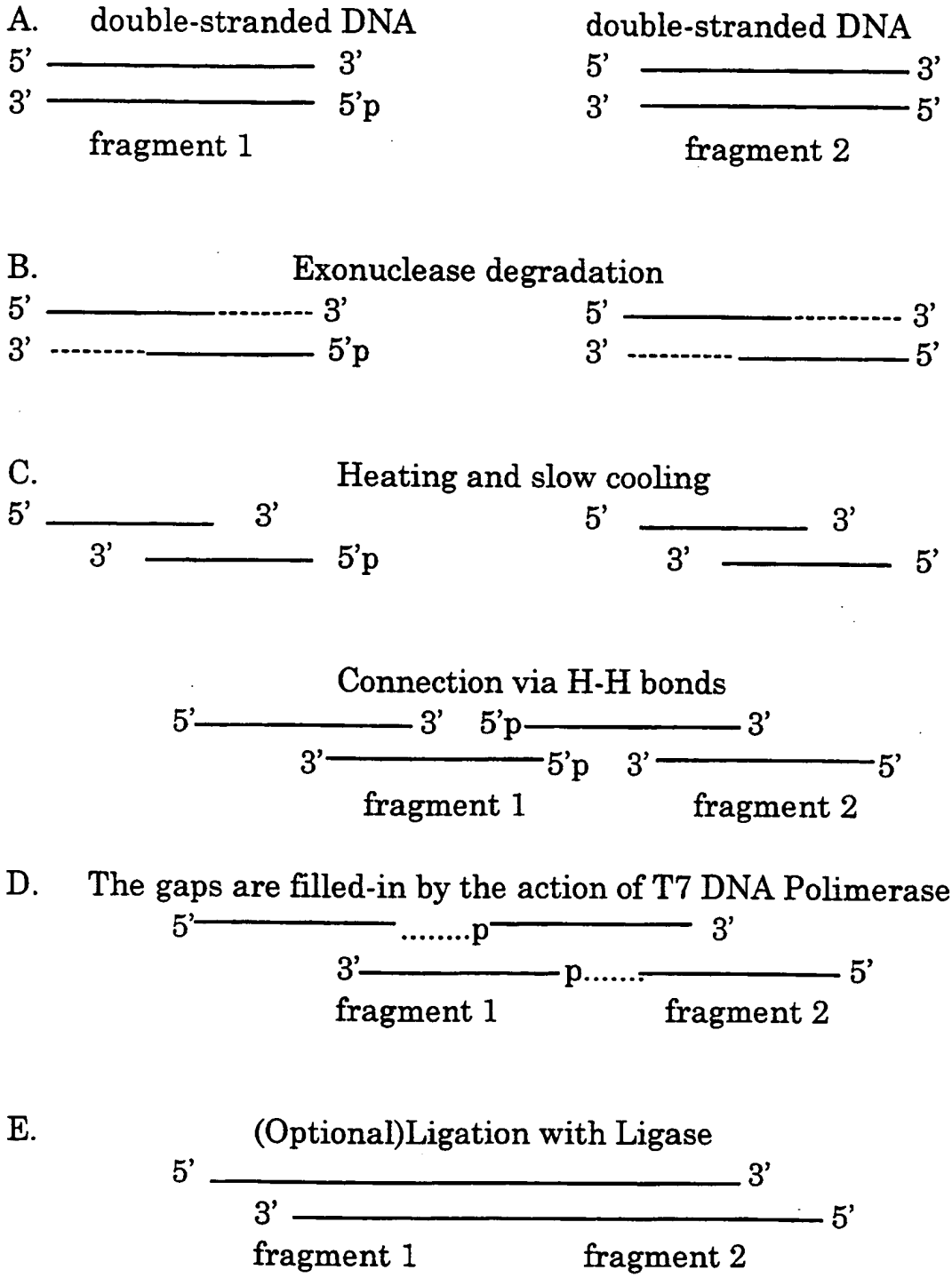
A.
double-stranded DNA
5' 3'
3' 5'p
fragment 1
double-stranded DNA
5' 3'
3' 5'
fragment 2
B.
Exonuclease degradation
5' 3'
3' 5'p
5' 3'
3' 5'
C.
Heating and slow cooling
5' 3'
3' 5'p
5' 3'
3' 5'
Connection via H-H bonds
5' 3' 5'p 3'
3' 5'p 3' 5'
fragment 1
fragment 2
D.
The gaps are filled-in by the action of T7 DNA Polimerase
5' .....p 3'
3' p..... 5'
fragment 1
fragment 2
E.
(Optional)Ligation with Ligase
5' 3'
3' 5'
fragment 1
fragment 2

Fig. 1

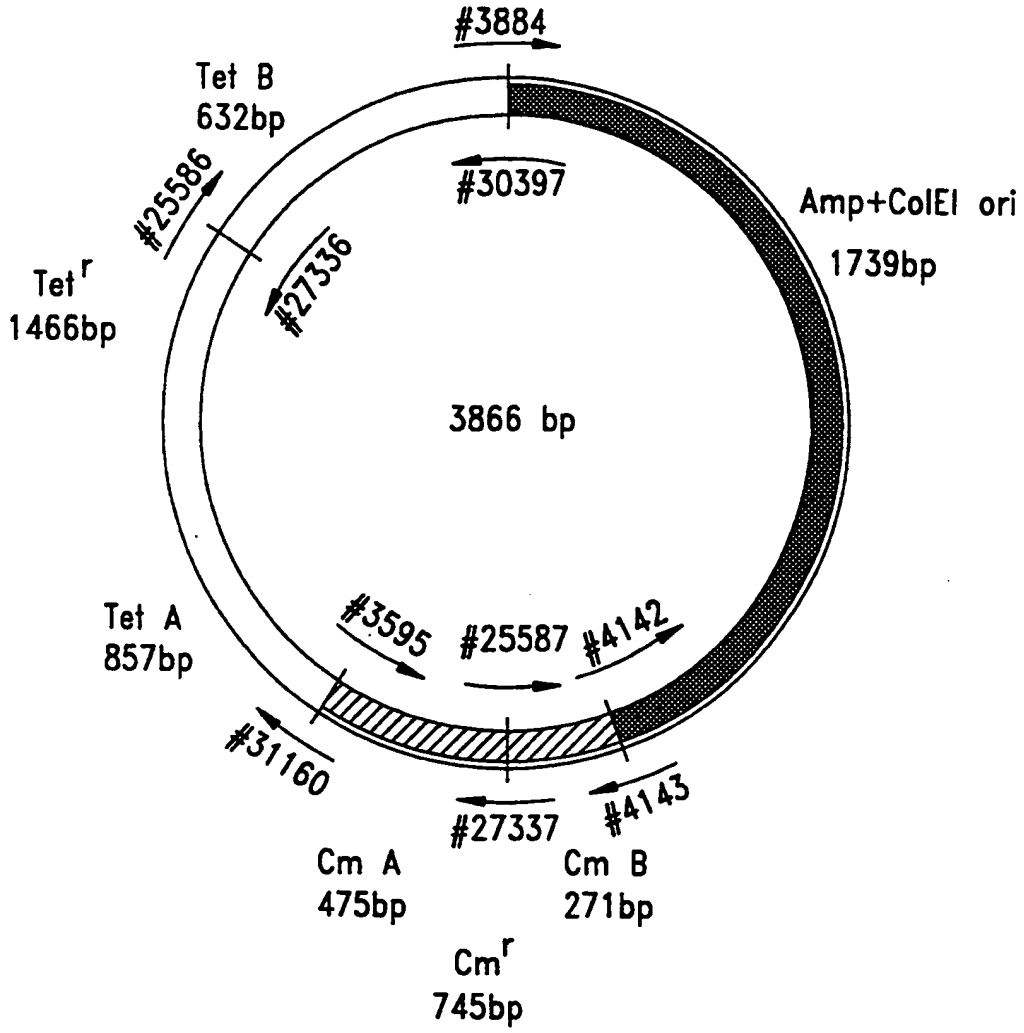
#3884
Tet B
632bp
#25586
#30397
Amp+ColEI ori
1739bp
Tet r
1466bp
#27336
3866 bp
Tet A
857bp
#3595
#25587
#4142
#31160
#27337
#4143
Cm A
475bp
Cm B
271bp
Cm r
745bp

Fig. 2

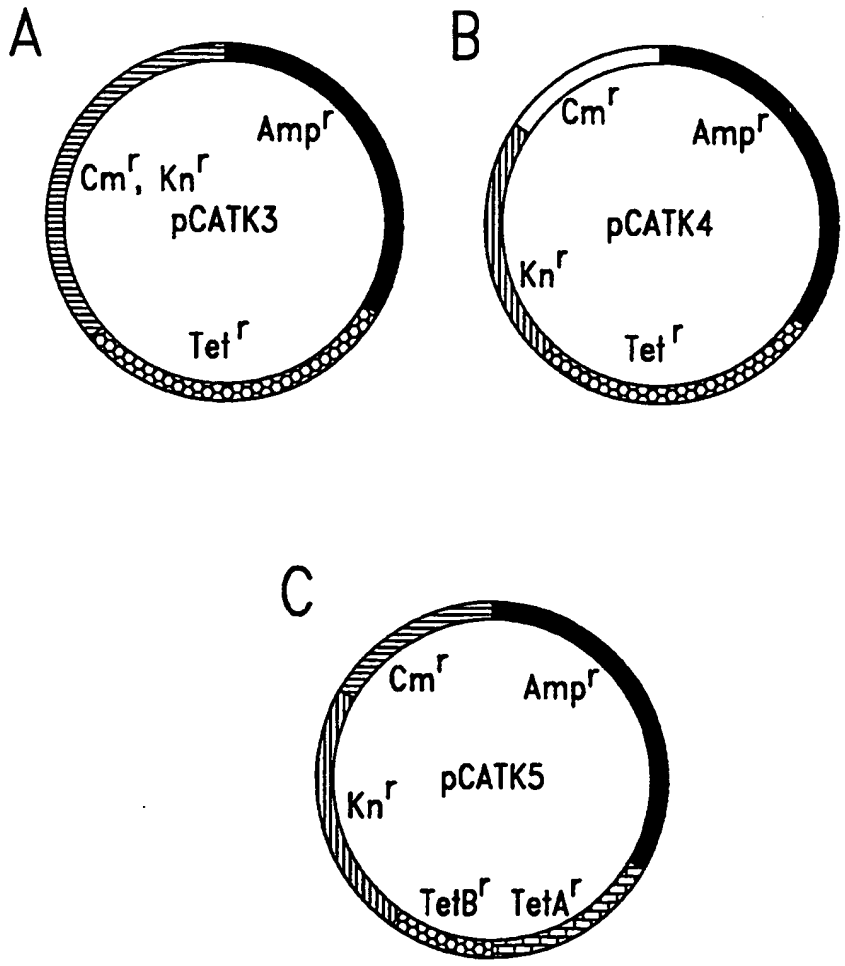
A
Amp^r
Cm^r, Kn^r
pCATK3
Tet^r
B
Cm^r
Amp^r
pCATK4
Kn^r
Tet^r
C
Cm^r
Amp^r
Kn^r
pCATK5
TetB^r
TetA^r

Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 98/00095

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12Q1/68 C12N15/66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOOTH P.M. ET AL.: "Assembly and cloning of coding sequences for neurotrophic factors directly from genomic DNA using polymerase chain reaction and uracil DNA glycosylase" GENE., vol. 146, no. 2, 1994, AMSTERDAM NL, pages 303-308, XP002071998	20
Y	see the whole document	1
A		5-12

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 August 1998	17/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 98/00095

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KALUZ S ET AL: "Directional cloning of PCR products using Exonuclease III" NUCLEIC ACIDS RESEARCH, vol. 20, no. 16, 1992, LONDON GB, pages 4369-4370, XP002072726 cited in the application	1
A	see the whole document	2,3,14, 15

X	SMITH C. ET AL.: "Generation of cohesive ends on PCR products by UDG-mediated excision of dU, and application for cloning into restriction digest-linearized vectors" PCR METHODS & APPLICATIONS., vol. 2, no. 4, May 1993, ING HARBOR LABORATORY PRESS US, pages 328-332, XP002071999	20
A	see the whole document	5-13,16, 19

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A	see abstract see page 1, line 52 - page 2, column 28 see figure 1 see page 7, line 45 - page 13, line 55 see examples 1-4,8-13	1-3,14, 15

X	RASHTCHIAN A. ET AL.: "URACIL DNA GLYCOSYLASE-MEDIATED CLONING OF POLYMERASE CHAIN REACTION-AMPLIFIED DNA: APPLICATION TO GENOMIC AND cDNA CLONING" ANALYTICAL BIOCHEMISTRY, vol. 206, no. 1, 1 October 1992, pages 91-97, XP000311343 see the whole document	20

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ational Application No
PCT/IL 98/00095

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		JP 3232489 A	16-10-1991
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